Comparative investigation of miRNAs in halophilic marine bacteria and barley plants

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by

Thi Hoang Yen Dang

Department of Chemistry and Biotechnology

Faculty of Science, Engineering, and Technology

Swinburne University of Technology

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Abstract

MicroRNAs (miRNAs) are post-transcriptional regulators that play a critical role in a number of physiological processes, including stress responses to changes to external environmental conditions. Since their first discovery in 1993, miRNAs have been investigated in plants, animals, and several pathogenic bacteria, while some limited research has been carried out using environmental bacteria. In this project, miRNAs in marine halophilic bacteria and barley plants were comparatively investigated. The sequence relatedness of miRNAs identified in both types of organisms was studied in order to investigate the origin, evolution and possible mechanism/s of the miRNA post-transcriptional regulation in plants and environmental bacteria.

Next-generation small RNA-Sequencing (small RNA-Seq) was employed to investigate the miRNA population in bacteria of the genus *Thalassospira*. Bacteria of this genus have displayed high adaptability to environmental changes, tolerating exposure to up to 10% (w/v) NaCl (approximately 1.7 M NaCl). The newly described species, *Thalassospira australica* NP 3b2^T, along with other validly published species of this genus, were employed in this study. The whole genome sequences of *T. australica* NP 3b2^T and other species of the genus *Thalassospira* allowed the identification of a complete set of miRNAs within the bacteria of this genus. A total of 984 putative miRNAs were identified from nine species of the genus *Thalassospira*. Of which, fifty seven conserved putative miRNA sequences were found in the studied species and six identical sequences were found at different locations of some species suggesting a characteristic to the genus miRNA sequences pattern. The high expression level of these putative miRNAs suggested that these miRNA may play specific physiological role in the growth and development of bacteria of this taxon.

Next-generation small RNA-Sequencing (small RNA-Seq) was used to identify and characterize the miRNAs that were responsive to salinity exposure in different barley genotypes, salt sensitive (Arivat barley) and salt tolerant (Calmariout barley) cultivars. The expression of these miRNAs was comparatively analysed in both barley cultivars. It was found that 231 miRNA types were expressed in the barley leaf, including 41 salt responsive miRNAs and 25 novel annotations that showed variation

in their expression between two distinct barley genotypes. A total of 68 transcripts were also identified as potential targets for these miRNAs. Some of the predicted targets, such as the transcription factor MYB (myeloblastosis), which is responsive to the stress hormone abscisic acid (ABA), and ubiquitin-conjugating enzyme, mitogen-activated protein kinase or serin/threonine-protein kinase, were identified to be involved in plant growth and/or stress regulation. Fifteen barley cultivars were assessed according to their relative water content and Na^+/K^+ ratio under acute stress conditions (150 mM NaCl for 6 h and 24 h). The results provided a significant variation in salt resistant level of varieties through the measurement of physiological response. Six selected genes, *i.e.*, squamosa promoter-binding protein (SPL), GRAS transcription factor (SCL), MYB6, choline monooxygenase (CMO), betaine aldehyde dehydrogenase (BADH) and ABA insensitive gene (ABI) were then investigated for their expression in response to salinity in eight barley cultivars varying salt resistance using real-time PCR. The MYB transcription factor was found to be the most differentially regulated among the tested genes during salt stress exposure, and therefore this factor could be a suitable salt tolerant candidate for the improvement of the growth of crops being cultivated in saline soils.

The comparative phylogenetic analysis of miRNAs in marine bacteria and barley showed a high level of relatedness (≥ 50%) of miRNAs in environmental prokaryotic organisms and plants based on homology to 5’ end seed sequence and full-length mature miRNA sequence criteria.

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Declaration

I, Thi Hoang Yen Dang, declare that this thesis is original work and contains no material that has been accepted for the award of Doctor of Philosophy, or any other degree or diploma, except where due reference is made.

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Signature 

List of publications

Publication arising from this thesis

Book chapter

1. **Dang, T. H. Y.,** Ziemann, M., and Bhave, M. (2013). Abiotic stress response in barley and the emergent roles of microRNAs. *In:* Barley: Physical Properties, Genetic Factors and Environmental Impacts on Growth. Hasunuma. K. (EDs) Nova Science Publishers, New York, pp 165-192

Peer-reviewed articles

1. Ivanova, E.P., López-Pérez, M., Webb, H.K., Ng, H.J., **Dang, T.H.Y**., Zhukova, N.V., Mikhailov, V. V., Crawford, R.J and Rodriguez-Valera, F (2016) *Thalassospira australica* sp. nov. isolated from sea water, *Antonie van Leeuwenhoek*, 109, 1091-1100

2. **Dang, T.H.Y**., Ziemann, M., Kamboj, A., Osta, A.E., Crawford, R., Ivanova, E.P and Bhave, M (2016) Analysis of salt responsive microRNAs and their targets in barley genotypes varying in salt stress response. *Acta Physiologiae Plantarum* (in preparation)

3. **Dang, T.H.Y**., Tyagi, S., D' Cunha, G., Bhave, M., Crawford, R and Ivanova, E.P (2017) Computational identification of microRNAs in marine bacteria of the genus *Thalassospira*. *Plos ONE* (submitted)

Conference proceeding

1. **Dang THY**, Kamboj A, Ziemann M and Bhave M (2014) Analysis of barley microRNAs under salinity stress using small RNA-seq In: *International Proceedings of Chemical, Biological & Environmental Engineering*, vol.70, pp.74-79, 9-11 June, Bangkok, Thailand

Conference and poster presentation with published abstract

1. **Dang, THY**, Ziemann M and Bhave M (2015) Salt stress responsive microRNAs and their potential targets in barley. Poster presentation at Lorne Genome Conference, 15-17 February, Victoria, Australia.

2. **Dang, THY**, López-Pérez, M, Crawford, RJ, Rodriguez-Valera, F and Ivanova, EP (2016) Genomic characteristics of Thalassospira australica isolated from sea water. Poster presentation at Molecular Microbiology Meeting Conference, 2-3 March, Sydney, Australia

Other publications

1. Wang, X., Bhadra, C.M., **Dang, T.H.Y.**, Buividas, R., Wang, J., Crawford, R.J., Ivanova, E.P and Juodkazis, S (2016) A bactericidal microfluidic device constructed using nano-textured black silicon. RSC Advances, 31, 26300-26306

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List of Abbreviations

ABI	ABA insensitive gene
AGO	Argonaute
AGRF	Australian Genome Research Facility
ANI	Average nucleotide identity
BADH	Betaine aldehyde dehydrogenase
BDT	Big Dye Terminator
BLAST	Basic Local Alignment Search Tool
bp	base pair (s)
cDNA	complimentary DNA
CMO	Choline monooxygenase
CSD	Cu/Zn superoxide dismutase
DCL1	Dicer Like-1
DDH	DNA-DNA hybridization
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
G+C	Guanine-cytosine content
GB	Glycine betaine
gDNA	genomic DNA
HEN1	Hua Enhancer 1
Hv	*Hordeum vulgare* (barley)
IBSC	International Barley Genome Sequencing Consortium
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani media
miRNA	microRNA
mRNA	messenger RNA
MUT68	a terminal nucleotidyltransferase
NCBI	National Center for Biotechnology Information

NGS	next-generation sequencing
NJ	Neighbour-joining
Os	*Oryza sativa* (rice)
PCR	polymerase chain reaction
Pre-miRNA	precursor microRNA
Pri-miRNA	primary microRNA
RBR	Relative binding ratio
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	Ribosomal RNA
siRNA	small interfering RNA
sRNA	small RNA
SSC	Saline sodium citrate
T_a	Annealing temperature
Ta	*Triticum aestivum* (common wheat)
TAE	tris acetate ethlenediaminetetracetic acid buffer
TF	Transcription factor
T_m	Melting temperature
UV	ultra-violet
X-gal	5-bromo-4-chloro-3-indolyl-b-D galactopyranoside

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Chapter 1.

Introduction

1.1 Overview

The discovery of the first microRNA (miRNA) in *Caenorhabditis elegans* opened up a new concept of regulation of gene expression at post-transcriptional level by small, non-coding RNA. The field of miRNA research has been rapidly expanded, numerous miRNAs have been identified in eukaryotic organisms. miRNAs were reported to play vital roles not only in normal growth and development processes (Bartel, 2004), but also in regulation of genetic responses to environmental factors in plants in particular (Sunkar and Zhu, 2004), as potential candidates for transgenic strategy in production of plants tolerant to severe environmental conditions.

Among environmental stressors, salinity is one of the most severe stresses that is detrimental to the growth and development of plants. Currently, salinisation occupies about 20% of world's cultivated area (Singh et al., 2015), being forecasted to salinise up to 50% of all arable land by 2050 (Wang et al., 2003). The increasing damage of salinisation, together with rapid expansion of human population prompted investigations toward discovery of novel genes involved in salt tolerance to ensure adequate food supply. While plant's miRNAs have been in focus of intensive research (Luan et al., 2015, Sunkar et al., 2006, Zhao et al., 2009, Long et al., 2015), the occurrence of miRNAs in environmental bacteria has not been studied. Due to their long-term survival in the presence of high salt concentrations, marine bacteria are regarded as most suitable candidates to study the genetic mechanisms that are evolved in response to salt tolerance.

In light of better understanding the evolutionary developed stress-responsive adaptations and biological functions of miRNA, it is of significant importance to study the phylogenetic relationships of miRNAs across prokaryotic and eukaryotic organisms. The conservation of miRNAs across different species belonging to animal and plant kingdoms was recently reported (Campo-Paysaa et al., 2011, Cuperus et al., 2011, Rathore et al., 2016). However, no similar studies have been undertaken using environmental bacteria.

1.2 Aims of the project

The primary aim of this project is to identify the novel genes that are involved in salt tolerance by using comparative analysis of miRNAs in halophilic bacteria and barley

plants. Both barley and marine bacteria are prospective organisms for the study of the genes responsive to salt tolerance due to well-known ability of these organisms to adapt to high salinity conditions. A series of tasks have been carried out to achieve the aim, as follows.

In the first intermediate aim, the comprehensive classification and identification of a novel species belonging to the genus *Thalassospira*, isolated from St. Kilda Beach, Port Philip Bay, Victoria, Australia, was identified using a combination of traditional techniques as well as whole genome sequence analysis.

The second intermediate objective was the identification of miRNAs population in bacteria of the genus *Thalassospira* was carried out using next-generation small RNA-Sequencing. The genus *Thalassospira*, currently housing ten valid species, reportedly tolerating up to 10% (w/v) NaCl (approximately 1.7 M NaCl). The complete identification of miRNAs within the genus would provide an evidence on the occurrence of miRNAs in environmental bacteria.

The third intermediate objective is the identification of miRNAs in barley genotypes varying in salt tolerance. The next-generation small RNA-Sequencing (small RNA-Seq) was employed to identify full-set of salt responsive miRNAs in barley which the miRNAs were further validated by cloning and DNA sequencing. The putative targets were also predicted that would provide a better understanding of miRNA expression and their targets for further application on genetic selection/ breeding.

The fourth intermediate objective was to evaluate the salt tolerance level of fifteen barley varieties, currently cultivated in Australia. Of the cereal crops, barley (*Hordeum vulgare L.*) is classified as the salt tolerant crop (Niazi et al., 1992). Unlike other cereals, barley can grow under adverse environmental conditions, such as drought, salinity, alkaline soil or cold as well as adapt to high altitude regions or even in deserts (Ullrich, 2011). Therefore, barley could be employed as a plant model to study the unique genetic traits that could be used for further improvement of other crops through the processes of genetic engineering.

In the context of this aim, key physiological assays including relative water content and Na^+/K^+ ratio were used for assessment of barley genotypes response to salt

stress over period of times. The results would provide good candidates for study of gene related to salt tolerance. A number of genes found in halophilic bacteria have similar functions in stress response to plants, e.g., trehalose, glycine betaine, heat shock like proteins or Na^+ and H^+ antiporters (Das et al., 2015, Rubiano-Labrador et al., 2015, Thombre et al., 2016). Following this, the expression patterns of six genes involved in salt tolerance were investigated in the leaf and the root of eight distinct salt tolerant barley cultivars. The real-time PCR technique was used to detect the differential expression of these genes that might be promising candidates for stress tolerance.

The last intermediate objective in this thesis was to comparatively analyse miRNAs in the bacteria of the genus *Thalassospira* and barley. This task involves the finding of conserved miRNAs in organisms belonging to two lineages. The results would contribute significantly to the fundamental knowledge on the evolution and origins of miRNAs across two kingdoms.

1.3 Thesis outline

The thesis is comprised of ten chapters. Chapter 1 overviews the global issues of salinization and the role of miRNA as new gene expression regulation for crop improvement. It also addresses the aim of the project on contribution of genes associated to stress tolerance with a series of designed tasks to achieve the aim. Chapter 2 provides critical review of the effect of environmental stress on plants, especially salinity, and the importance of barley as the salt tolerant plants. A comprehensive review on miRNAs as ideal candidates for the search of the stress-responsive genes and the existence of miRNA in bacteria are also provided. Chapter 3 describes the materials and methods adopted in this study. Chapter 4 provides the description of a novel species of the genus *Thalassospira* isolated from sea water which will be included in the following Chapter 5, which describes the identification of miRNAs in all valid species of the genus. In chapter 6, the identification of full-set miRNAs was undertaken. The salt responsive miRNAs were also detected and their putative targets were further predicted. Chapter 7 evaluates the salt tolerance level in fifteen barley cultivars based on the physiological approach, which takes into account relative water content and Na^+/K^+ ratio as critical criteria. Chapter 8 investigates the response of reported salt responsive genes on barley genotypes varying salt stress. Chapter 9 provides an analysis of miRNAs found in both bacteria of

the genus *Thalassospira* and barley plants. Chapter 10 summarises the major findings of this project and suggests further directions.

Chapter 2.

Literature review

2.1 Overview

This chapter presents a review of literature on the presence, biogenesis and function of the recently discovered regulators of gene expression such as the small non-coding RNAs, microRNA (miRNA) in barley and bacteria. In the first part, a summary of the barley *Hordeum vulgare* L. agricultural industries will be provided, focusing on its importance as food and as an industrial crop. The role of miRNA in the response to abiotic stress as well as their target genes will be discussed. Further, an overview of the occurrence of miRNA-sized in bacteria will be provided. A discussion on the development of the RNA sequencing techniques, in addition to the bioinformatics tools that could be used for the identification and characterization of miRNA, will conclude the literature review.

Part I miRNA in barley

2.2 Barley: origin and importance

Barley *Hordeum vulgare* L. is one of the most economically important crop species in the world, being ranked fourth behind maize, rice and wheat. Barley has been cultivated over 491 million hectares and more than 143 million tonnes was produced world-wide in 2013 (FAO 2015, http://faostat3.fao.org/download/Q/QC/E). Barley belongs to the grass family *Poaceae* subfamily *Pooideae*, and tribe *Triticeae* (Gaut, 2002). *Pooideae* was estimated to have originated some 46 million years ago (MYA). The *Triticeae* (barley and wheat) probably diverged from oats 25 MYA and barley diverged from the common ancestor with wheat almost 13 MYA (Figure 2.2.1). Historically, barley has been used in the wild form of *Triticeae* species before domestication and cultivation (Zohary and Hopf, 2000).

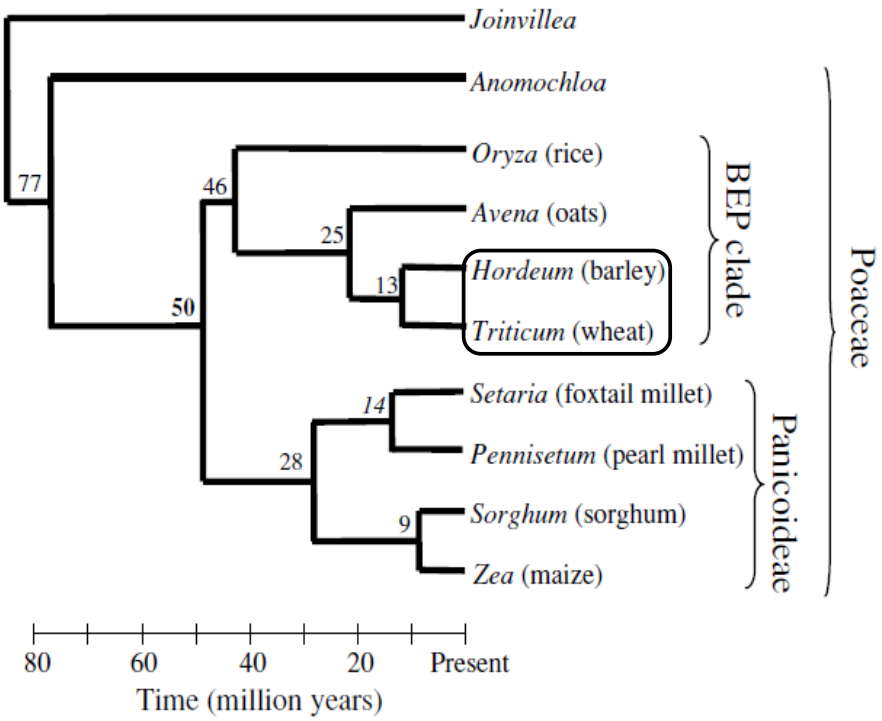

Figure 2.2.1 Phylogenetic relationships of grass species (adapted from Gaut (2002). Evolutionary tree shows the location of barley and wheat. The *Triticeae* tribe is highlighted with a black rounded rectangle.

2.2.1 Origin and evolution of cultivated barley

Barley *Hordeum vulgare* L. is one of the original crops of Old World agriculture. Archaeological and molecular evidence has proven that domestication of barley took place about 10,000 years ago at various sites in the Fertile Crescent (an ancient area of fertile soil and important rivers from the Nile to Tigris and Euphrates rivers) (Zohary and Hopf, 2000), possibly through environmental or human selection (Bothmer et al., 2003). Genetic evidence has been found that barley *Hordeum vulgare* L. was domesticated on more than one occasion. The first domestication in the Fertile Crescent possibly led to the significant diversity in European and American cultivars, while the second domestication in the same region contributed to the diversity in barley from Central Asia to the Far East (Morrell and Clegg, 2006). The *Hordeum spontaneum* and *Hordeum vulgare* (*H. vulgare*) barleys are morphologically similar, with the cultivated form *H. vulgare* developing tough, rachis, six-rowed spikes, less brittle and naked caryopsis (Salamini et al., 2002). Cultivated barley is diploid (HH; 2n = 2x = 14), and was originally used for human

consumption for approximately 8,000 years before the common era (BCE) throughout North Africa, Southern Europe, East Asia, and North and South America. Over the years it has evolved mainly into a feed grain, but also used for brewing beer (Newman and Newman, 2008). Despite having undergone different evolutionary processes, genetic evidence has shown that wild populations possessed genetic differences that have not changed significantly since barley was first domesticated (Bard et al., 2000).

2.2.2 Importance of barley as crop

Barley can be grown in regions with adverse environmental conditions, such as deserts or high mountains (>4,500 m on the Altipano of Peru and Bolivia) (Ullrich, 2011), under marginal conditions such as drought (Lopezcastaneda and Richards, 1994), alkaline soil (Murata et al., 2006) or frost (Plotnikov et al., 2012), conditions to which other cereals have not adapted well (Ullrich, 2011). Hence, an understanding of the unique genetic traits of barley could be useful in efforts to further improve other crops via the processes of selective breeding and genetic engineering.

The barley grain has a highly nutritive composition of carbohydrates (80%0, protein (7-25%) and lipids (3%) (Newman and Newman, 2008). Barley is a good source of vitamins, especially vitamin B3, at levels four to five times higher than that of maize, oats and rye (Baik et al., 2011) and vitamin E (Kerckhoffs et al., 2002). It also contains phytochemicals and minerals such as potassium, calcium, phosphorus, iron, magnesium and zinc (Newman and Newman, 2008). Barley is also a functional food and has been reported to reduce blood cholesterol, assisting in the prevention of cardiovascular disease, hypertension, stroke as well as diabetes (Newman and Newman, 2008). Although it is now used mainly for animal feed (75%), malt (20%) and as an ingredient of food products (5%) (Ullrich, 2011), it is still considered a major food in parts of Asia and North Africa.

Barley is one of the most important crops worldwide, ranking fourth highest cultivated cereal crops. It is typically cultivated over 49 million hectares of arable land and produces >143 million tonnes annually (Food and Agriculture Organisation, 2015). In Australia, barley is one of the most important grain crops due to the quantity produced, the area cultivated and the revenue generated. From 1961 to 2011, barley yields have more than doubled (0.98 t/ha in 1961 to 2.18 t/ha in 2011). Australian barley has an annual production of 7 million tonnes per year, occupying almost 4 million harvested

hectares (Barley Australia 2015) from Western Australia to Southern Queensland. Australia has a reputation for high-quality of barley production and is a leading exporter of barley, with 4 million tonnes exported each year (Barley Australia 2015). The trade value of barley for the 2014 season was A$ 2.199 billion, (ABARES 2014). Thus, while the contribution of barley production to Australia's national economy is significant; its production is under threat from severe abiotic stresses.

2.3 Barley genome sequencing projects

The cultivated barley *H. vulgare* L. has a diploid genome (2n=14). It has a 5.1 gigabase (Gb) genome that has been used widely to study genetic variation since the late 1920s. The functional and structural genomics of barley has been studied extensively over the last decade, providing 478,734 public ESTs (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html) with 50,000 tentative unigenes (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=barley) (Sreenivasulu et al., 2008). The International Barley Genome Sequencing Consortium (IBSC) was established in 2006 to achieve a high quality reference genome sequence and a complete physical map (Schulte et al., 2009). The Roche 454 sequencing technology was initially employed for testing on four barley Bacterial Artificial Chromosome (BAC) clones (Wicker et al., 2006), initially for sequencing the flow-sorted chromosome 1H (Mayer et al., 2009), then all other chromosomes (Mayer et al., 2011). The draft whole genome project was released in October 2012 and the data is now available at the International Barley Genome Sequencing Consortium (http://barleygenome.org; last accessed April 2015). Barley is considered as one of the best-studied systems in cereal crops and a general model for Poaceae seed development and germination, due to its significant contribution to the malting and brewing industry and orthology to rice and wheat genomes (Schulte et al., 2009). 50% of barley genes are highly homologous to rice due to these grains sharing their last common ancestor about 70 million years ago (Stein et al., 2007). Due to its simple diploid genome, it is also a good genomic model for hexaploid bread wheat (*Triticum aestivum* L.). The release of the barley genome thus provides significant genomic and transcriptomic resources towards gene discovery and varietal improvement (by selection and breeding, or transgenic technologies), for increased yields and tolerance to environmental stresses.

2.4 Abiotic stresses limiting the growth of barley

Salinity, drought or extreme temperatures are the major stressors that limited the significant growth, quality and production of crop species worldwide. Historically, warming and shifts in rainfall have caused severe agricultural losses (US $10.3 billion in the United States in 2011 (U.S. Department of Agriculture's Risk Management, 2012)). Adverse climate change conditions have affected the ecology and agricultural distribution of barleys (Schmidhuber and Tubiello, 2007). Soil and water salinity concerns have been reported in many countries such as China, India, the United States, Argentina, Sudan, Western and Central Asia, with salinisation of nearly 34 million irrigated hectares (FAO, 2011). To cope with this type of adverse stressor, research on plants capable of adapting to these conditions, as well as selection/propagation of cultivars showing tolerance, has been promoted by the Food and Agricultural Organization (FAO) of the United Nations (FAO, 2007) and the Global Partnership Initiative for Plant Breeding Capacity Building (GIBP) (GIPB, 2012) in an effort to improve food production and agriculture.

The most significant abiotic stresses that limit the growth of barley are soil salinity and drought. Soil salinity, and the associated ground water salinity, is a global issue, currently affecting about 20% of world's cultivated area (about > 900 million hectares) (Singh et al., 2015). These increases in salinity may lead to losses of up to 50% of all arable land by 2050 (Wang et al., 2003). Currently, 5.7 million hectares in the major agricultural regions of Australia have already been affected, with another 17 million hectares (1/3 of the available agricultural land) being forecast to become salinised by 2050 (Rengasamy, 2010) if no efforts are made to combat the spread of this salinity. Salinity can be categorized into two main types: primary and secondary salinity. Primary salinity arises from natural sources such as soils and waters, whereas secondary salinity usually arises due to human activities, e.g., land development and agriculture. Secondary salinity usually occurs due to irrigation, dry land, sea water intrusion and point source (Queensland Government 2013, https://www.qld.gov.au/environment/land/soil/salinity/types/). Secondary salinity is one of the most severe threats to the ecosystem in Southern Australia, and is predicted to affect more than 2 million hectares of native vegetation in Australia by 2050 (Cramer and Hobbs, 2005). A high concentration of salt in soil prevents the uptake of water through plant roots, and hence is detrimental to the growth of the plant. Generally speaking, plants

respond to salinity in two phases i.e. the osmotic (phase I) and ion-specific (phase II) (Munns and Tester, 2008). The osmotic phase is an initial response to salinity when water becomes less accessible to the roots, leading to reduced leaf and root growth (Munns, 1993). Phase II starts later when the salt accumulates to toxic levels, resulting in leaf dehydration and eventually death of the plant (Munns and Tester, 2008). Salinity is known to influence the growth and development of plants by (i) inhibiting the activity of nucleic acid metabolism enzymes (Gomes-Filho et al., 2008), (ii) impacting various processes involved in germination such as seed imbibition (Wahid et al., 2010), (iii) reducing the amount of photosynthesis and cell growth in the plant due to a reduction in the availability of CO_2 (Chaves et al., 2009) and (iv) increasing the formation of reactive oxygen species (ROS) such as O_2, H_2O_2, $O_2^{\cdot}$, $HO^{\bullet}$, that cause oxidative damage to the plant proteins, DNA and lipids (Gill and Tuteja, 2010). The adverse effects of environmental stresses on plant growth and crop yield can lead to a shortage of available food resource for human populations.

Drought stress also severely impedes the production of barley and other crops (Moffat, 2002). Drought occurs when there is limited rainfall, increased evaporation and the presence of non-wetting or poorly-wetting soils, limiting the extent of water storage that can take place in the soil (Wery et al., 1993). The severity of drought is, therefore, unpredictable, causing an adverse impact on physiological processes such as stomatal conductance, lipid accumulation, photosynthesis and gene expression (Rizhsky et al., 2004). The effects of water deficit have been widely studied that result in (i) cell dehydration, (ii) increased production of reactive oxygen species (ROS) causing oxidative damage to enzymes and cellular structures, (iii) stomatal closure leading to reduced CO_2 uptake (Gill and Tuteja, 2010), and (iv) severely reduced grain yield by affecting pollen development (Dolferus et al., 2011). In Australia, rainfall deficiency has been reported to have remained constant or slightly increased from July 2014 to July 2015, notably in Tasmania, Western Australia, Queensland and Western to Central Victoria (Australia Government, Bureau of Meteorology, 2015).

Plants also require an optimum temperature for normal growth and development, and extreme conditions can cause severe stress effects in un-adapted plants. Chilling prevents water absorption, while freezing increases cell dehydration, leading to osmotic stress and hyper-accumulation of ROS. Exposure to high temperatures also results in

significantly reduced yields of barley (Bavei et al., 2011). Barley, together with wheat, is also susceptible to boron and aluminium toxicity. Boron is an essential micronutrient, important for the structures of cell membranes and walls, and its deficiency results in stunted growth (Dell and Huang, 1997). Boron is, however, toxic to barley at higher levels, and some areas of South Australia (about 5 million ha of arable land) contain toxic levels of boron (Schnurbusch et al., 2010). Aluminium toxicity is prevalent in acidic soils and about 50% of the world's arable land is estimated to be acidic (Wang et al., 2006). Aluminium stress in barley has been shown to affect the uptake and translocation of a number of minerals including phosphorus, calcium, magnesium and copper (Ali et al., 2011).

Given the detrimental effects of abiotic stresses on plant physiology, it is essential to develop an understanding of the innate mechanisms associated with stress tolerance in plants. In addition, gene technologies could be used, if appropriate, to ensure the productivity of grain crops. Plant responses to abiotic stresses have been widely studied, with a significant amount of literature now available on a number of genes, regulatory pathways and signaling mechanisms that function in diverse ways such as salt uptake and transport, osmotic balance, redox balance, chaperone activities and other protective functions (Munns and Tester, 2008, Hirayama and Shinozaki, 2010, Atkinson and Urwin, 2012, Baldoni et al., 2015, Singh et al., 2015). Some of the main features of plant responses to abiotic stress will be summarized below.

The change in expression of some genes has been investigated as plants have been exposed to abiotic stresses. These genes are involved in signal transduction pathways such as transcriptional factors (ABF/ABARE, CBF/DREB, WRKY families) (Nakashima et al., 2009) or in the accumulation of compatible solutes, such as sugars (trehalose), sugar alcohols (mannitol), amino acids (proline) and amines (glycine betaine) that can adjust osmotic pressure changes and scavenge ROS (Peleg et al., 2011). ROS production is increased by the synthesis of ROS scavenging enzymes, such as catalases (CAT), superoxide dismutase (SOD) and other non-enzymatic antioxidant molecules such as ascorbic acid during abiotic stress (Gill and Tuteja, 2010). Expression of other encoding genes that are involved in the protection of the macromolecules and membranes, such as late embryogenesis abundant (LEA) proteins (Olvera-Carrillo et al., 2011), heat shock proteins (Hsps) (Wang et al., 2004) have also been identified. Abcisis acid (ABA), a

phytohormone, has also been shown to play an important role in plants responding to abiotic stress by regulating stomatal closure and reducing the amount of water loss that takes place through transpiration processes (Cutler et al., 2010). In response to abiotic stress, a number of responsive genes have been detected that are involved in water and ion uptake, such as aquaporins (Tyerman et al., 1999), high-affinity potassium (K^+) transporters (HKT) (Hauser and Horie, 2010) and ion transporters (Blumwald, 2000), which exhibit a significant ability to maintain the osmotic balance under dehydration stress conditions. Among the recently discovered regulators of gene expression are the small non-coding RNAs, microRNA (miRNA). miRNAs can result in degradation of target coding mRNAs, causing gene silencing by prevention of translation. The regulation of genes in plants by small RNAs has been reviewed (Kumar, 2014, Zhang, 2015). The main focus of the present study is determining how miRNAs regulate the target genes during an abiotic stress response. The next sections provide a comprehensive review of plant miRNAs and their role in response to abiotic stress.

2.5 Discovery of miRNAs

miRNAs are endogenous, typically 19-25 nucleotides long, single-stranded, non-coding RNAs found in both animal and plant transcriptomes, and have roles in post-transcriptional regulation of gene expression through regulation of target mRNAs. The similar structure and function of miRNA was also observed in bacteria and will be discussed in detail later (Part II). The first miRNA, *lin-4*, was discovered by Lee et al. (1993) in *Caenorhabditis elegans* as a small, non-coding RNA with two transcripts of approximately 22 and 61 nucleotides. It was found to play an important role in timing control of larval development by regulating a target gene, *lin-14*, reducing the transcript levels of the latter by sequence complementarity between *lin-4* and the 3'UTR of *lin-14* mRNA. This discovery opened up a new concept of post-transcriptional regulation of gene activity by small RNAs. Reinhart et al. (2002) cloned small RNAs in *Arabidopsis thaliana,* and 16 of these matched the features of miRNA. They had different expression levels in seedlings, leaves, stems, flowers and siliques. Later on, numerous miRNAs were identified in diverse algae, e.g., 19 miRNAs in the green alga *Chlamydomonas reinhardtii* (Zhao et al., 2007), 26 in the brown alga *Ectocarpus* (Cock et al., 2010) and 231 in the red alga *Porphyra yezoensis* (Liang et al., 2010), suggesting that they may also be involved in gene regulation events in plants, considering the algal origin of plant

chloroplast. To date, miRNAs have been identified in diverse fungi, plants and animals and deposited in miRBase v21.0 (http://www. mirbase.org). MiRNAs appear to have evolved into family members from ancestral to related species of organisms. Cloned miRNAs from *C. elegans* and *C. briggsae* (Lim et al., 2003a, Lim et al., 2003b), or human and mouse (Lagos-Quintana et al., 2003), have shown miRNA conservation between them. Twenty miRNA families were also found to be conversed between *A. thaliana*, *Oryza sativa* and *Populus trichocarpa* (Jones-Rhoades et al., 2006), and many also amongst barley, wheat, maize, sorghum, *Brachypodium* and *Populus* (Schreiber et al., 2011, Yao et al., 2007). Many appear to be encoded by multiple genes (Table 2.5.1).

Table 2.5.1 Conserved miRNA families in plants with sequenced genomes.

miRNA family	**Sequence (5'-3')**	***Oryza***	***Hordeum***	***Triticum***	***Brachypodium***	***Arabidopsis***	***Populus***	***Maize***	***Sorghum***	**Ref.**
miR156	UGACAGAAGAGAGUGAGCAC	12	2	4	10	12	11	3	3	(2,4,6)
miR159/ 319	UUUGGAUUGAAGGGAGCUCUG	8	2	11	12	6	15	3	3	(2,4,6)
miR160	UGCCUGGCUCCCUGUAUGCCA	6	2	2	5	3	8	1	1	(2,4,6)
miR162	UCGAUAAACCUCUGCAUCCAG	2	0	0	0	2	3	0	0	(2,6)
miR164	UGGAGAAGCAGGGCACGUGCA	6	3	3	3	3	6	1	1	(2,6)
miR165	UCGGACCAGGCUUCAUCCCCC	0	0	1	0	1	0	0	0	(6)
miR166	UCGGACCAGGCUUCAUUCCCC	12	4	2	8	9	17	2	2	(2,4,6)
miR167	UGAAGCUGCCAGCAUGAUCUA	10	4	5	6	4	8	3	3	(2,4,6)
miR168	UCGCUUGGUGCAGAUCGGGAC	2	6	3	3	2	2	2	2	(2,4,6)
miR169	CAGCCAAGGAUGACUUGCCGA	17	5	10	9	14	32	5	5	(2,4,6)
miR171	UGAUUGAGCCGUGCCAAUAUC	9	2	8	5	4	10	3	3	(2,4,6)
miR172	AGAAUCUUGAUGAUGCUGCAU	4	0	6	6	5	9	3	3	(2,4,6)

miRNA family	**Sequence (5'-3')**	***Oryza***	***Hordeum***	***Triticum***	***Brachypodium***	***Arabidopsis***	***Populus***	***Maize***	***Sorghum***	**Ref.**
miR390	AAGCUCAGGAGGGAUAGCGCC	1	1	1	1	3	4	0	0	(2,4,6)
miR393	UCCAAAGGGAUCGCAUUGAUC	2	1	3	3	2	4	1	1	(2,4,6)
miR394	UUGGCAUUCUGUCCACCUCC	1	0	0	1	2	2	0	0	(1,2,4)
miR395	CUGAAGUGUUUGGGGGAACUC	23	1	2	3	6	10	0	0	(1,2,4)
miR396	UUCCACAGCUUUCUUGAACUG	5	3	6	6	2	7	1	1	(2,4,6)
miR397	UUGAGUGCAGCGUUGAUGAA	2	2	3	4	2	3	0	0	(2,4,6)
miR398	UGUGUUCUCAGGUCGCCCCUG	2	0	2	2	3	3	0	0	(1,2,4)
miR399	UGCCAAAGGAGAAUUGCCC	11	4	2	5	6	12	1	1	(2,4,6)
miR408	CUGCACUGCCUCUUCCCUGGC	1	0	1	2	1	1	1	0	(2,4,6)
miR437	AAAGUUAGAGAAGUUUGACUU	1	0	1	0	0	0	1	0	(5,6)
miR444	UUGCUGCCUCAAGCUUGCUGC	1	2	1	0	0	0	0	0	(2,4,6)
miR827	UUAGAUGACCAUCACGCAAACA	1	1	0	0	0	0	0	0	(3,4)

The numbers in each column indicate the number of identified genes for each miRNA in the respective sequenced genome.Underline indicates seed sequence. References (Ref.): (1): Gao et al. (2012); (2): Jones-Rhoades et al. (2006); (3): Lacombe et al. (2008); (4): Schreiber et al. (2011); (5): Sunkar et al. (2005); (6): Yao et al. (2007).

2.6 Some terminology and definitions related to miRNAs

2.6.1 Terms related to miRNA structure

The term 'primary miRNA' (pri-miRNA**)** refers to the long stem-loop (hairpin) structure, i.e., two sequences that complement each other within the same strand, the paired area forming the stem and the unpaired bases forming the loop. The 'precursor miRNA' (pre-miRNA) is a short stem-loop structure of about 64-303 nucleotides that is cut from pri-miRNAs, with the mature miRNA sequence usually located in the stem (Bartel and Bartel, 2003, Xie et al., 2005). The miRNA genes have their own promoter and typical TATA-box motif. In plants, they are usually monocistronic, located in intergenic regions or unannotated loci (Naqvi et al., 2012).

The term 'miRtron' refers to a group of introns encoding mature miRNAs. These introns are spliced out, forming hairpin structures and passing the dicing process (Ruby et al., 2007) (explained later). In plant, the first putative mirtron, osa-MIR1429, was identified in rice (Zhu et al., 2008). After this study, a number of mirtron were uncovered in model plants such as *Arabidopsis thaliana* and rice, with two studies being performed at the same time in 2012. Joshi et al. (2012) identified 16 mirtron candidates from rice using MirtronPred web server (http://bioinfoicgeb.res.in/mirtronPred) while Meng and Shao (2012) discovered 5 and 18 mirtron candidates from *Arabidopsis* and rice, respectively, via high-throughput sequencing (HTS) data and structure-based approach. In addition, mirtrons were also reported in non-model plants such as cassava (1) and foxtail millet (2) (Yi et al., 2013a, Patanun et al., 2013).

The term 'seed sequence' refers to the six nucleotides at positions 2-7 in a miRNA. This plays an important role in miRNA function, as miRNAs regulate their target mRNAs by recognising and binding to them via complementarity of the seed sequence to mRNA (Lewis et al., 2005). The strand complementary to miRNA in the duplex (Figure 2.6.1), or the antisense miR strand**,** is often called miRNA* (i.e., star sequence). The miRNA and miRNA* strands are also called miRNA5p and miRNA3p, respectively, to indicate the 5' and 3' strands of the encoding DNA, e.g., hvu-miR168-5p/3p or hvu-miR171-5p/3p indicating the strands located at 5' and 3' of stem-loop structure (Kruszka et al., 2013). The miRNA* strand was initially thought to be discarded or degraded when the miRNA-miRNA* duplexes were loaded into RISC complexes (Mourelatos et al.,

2002) (described below). Czech et al. (2009) showed, however, that it can be also incorporated with AGO2 in *Drosophila*, and takes active roles in gene silencing pathways.

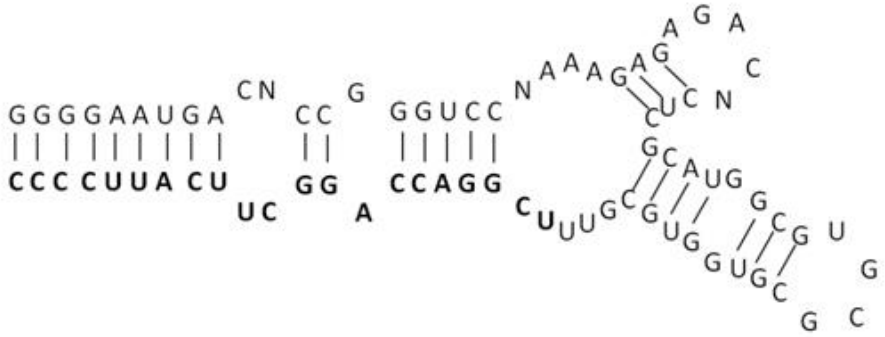

Figure 2.6.1 Stem-loop structure of barley pre-miR166m. The miR166m sequence is shown in bold.

2.6.2 miRNA families

miRNAs are generally considered to belong to the same family when there is significant similarity (difference of only one or two nucleotides) in the sequences of the mature miRNAs, and often identity in their seed sequence. The names of miRNAs ending with suffixes a, b, etc. generally indicate they belong to the same family. In some cases, however, they may not belong to a family, yet share sequences due to being transcribed by polycistronic genes or miRNA clusters, with different mature miRNAs being processed from one transcript. The suffixes may also indicate miRNAs expressed from opposite strands of pre-miRNA, or sense-antisense pair. These may thus have similarity in their respective 5' to 3' sequences; however, they cannot be processed from the same transcript and do not function together (Kruszka et al., 2013).

2.6.3 IsomiR

The term IsomiR refers to a group of miRNAs that vary at 5' and/or 3' ends. This concept came from Morin et al. (2008), who applied Illumina deep sequencing to identify miRNAs in human embryonic stem cells and found that several miRNAs exhibited abundant variants. IsomiRs are produced by imprecise excision of Drosha and Dicer within the pre-miRNA (Morin et al., 2008) (mechanism of miRNA biogenesis described

later). Based on the variations, they are classified into 5' isomiRs, 3' isomiRs and polymorphic isomiRs (Figure 2.6.2). The 3' isomers are found commonly in animals and plants, while the 5' isomer and polymorphic classes are rarely observed but hold significant proportion in the miRNA populations that they are present in (Neilsen et al., 2012). The 5' and 3' isomer classes each appear to be generated by exoribonucleases (which shorten the miRNA sequences), and nucleotidyltransferases (which extend the miRNAs) (Martin and Keller, 2007). Polymorphic isomiRs are likely produced by RNA editing, which can occur either in pri-miRNAs or pre-miRNAs. The editing typically involves substitution of adenosine by inosine (A-I) in double-stranded RNAs by 'adenosine deaminase acting on RNA' (ADAR) enzymes (Nishikura, 2010). Further studies (Cloonan et al., 2011) suggest isomiRs can regulate mRNAs based on their successful association with both the RISC complex (see later) and polysomes in the translation process. The properties of isomiR such as stability, incorporation efficiency and target modulating capacity are, however, unclear (Neilsen et al., 2012).

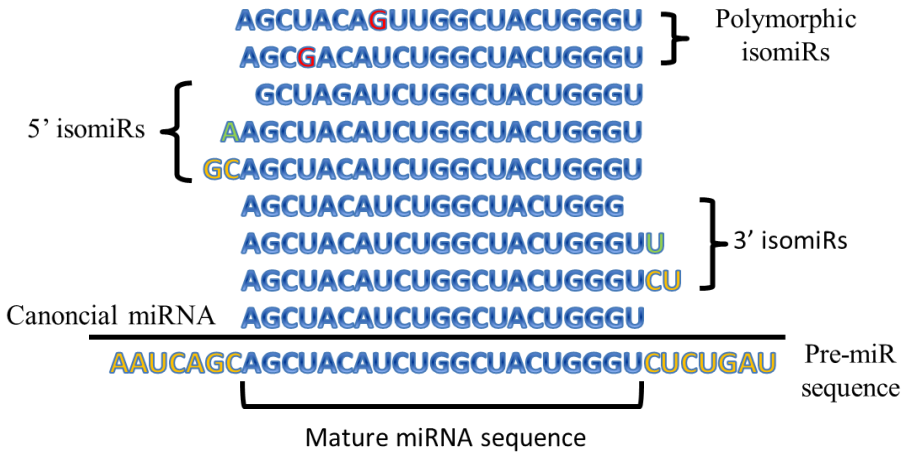

Figure 2.6.2 isomiRs of human miR222. Mature miRNA is highlighted in blue. 5' and 3' isomiRs from mature miRNA are produced by deleting or adding nucleotides to the 5' and 3' end of mature miR222, respectively. These isomiRs can be either template or non-templated variants. Polymorphic isomiRs contain a substituted nucleotide (red color) within a miR222 sequence.

2.7 Biogenesis of miRNAs

2.7.1 Transcription of precursor miRNA

In plants, mature miRNAs are encoded by their own genes. MiRNA genes are transcribed by RNA polymerase II into primary-miRNAs (pri-miRNA), which then undergo 5' capping and 3' polyadenylation (Xie et al., 2005). Genes of some miRNAs overlap with protein-encoding genes, wherein pri-miRNAs usually reside in the introns (Figure 2.7.1). In this case, pri-miRNA is produced by splicing of the intron, followed by maturation of miRNA from the spliced-out intron (Brown et al., 2008).

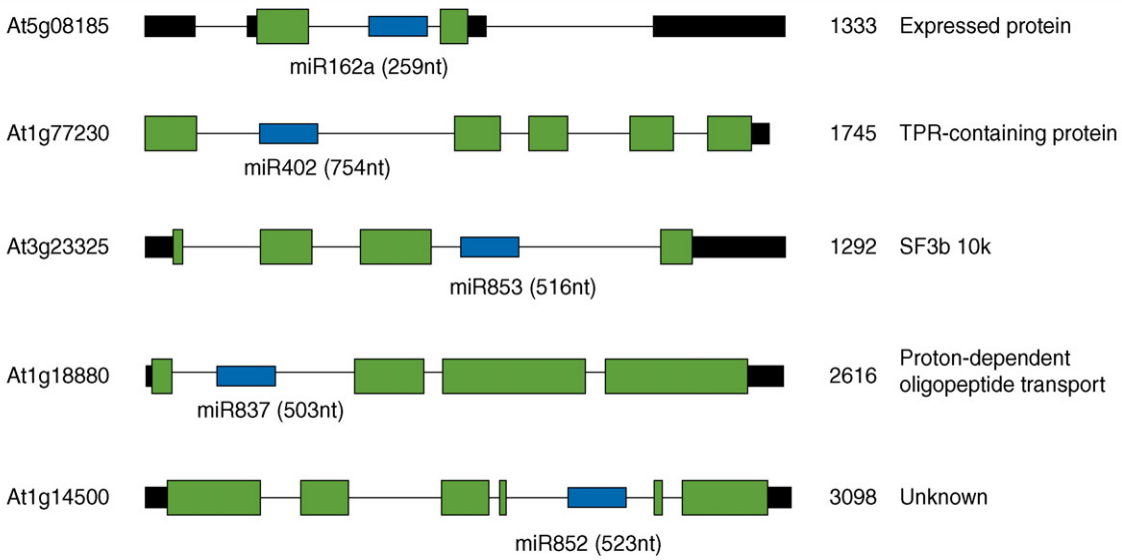

Figure 2.7.1 Examples of intronic miRNAs in *Arabidopsis* (adapted from Brown et al. (2008). The *Arabidopsis* (At) gene, length of transcript and gene function are given. MiRNA genes are located in the intron regions. Black box indicated 3' and 5' UTR exons; black lines: intron; blue box: miRNA and green box: exon

2.7.2 miRNA processing and export to cytoplasm

A pri-miRNA may contain one or more miRNA sequences within pre-miRNA molecules (Figure 2.7.2). In *Arabidopsis*, the processing of pri-miRNA to pre-miRNA(s) occurs in the nucleus, with the help of a 'microprocessor', an enzyme complex that includes Dicer Like-1 (DCL1), an RNAse type III enzyme, the Hyponastic Leaves 1 (HYL1), a dsRNA-binding protein, and enzymes such as C2H2 zinc-finger protein SERRATE (SE) and nuclear CBC, assisting to make the activity of DCL1 more efficient (Bologna et al., 2013) (Figure 2.7.2). DCL1 then makes another cut, now in pre-miRNA,

to liberate the miRNA together with its reverse complement, forming the miRNA-miRNA* (or miRNA5p-miRNA3p) complex (Bologna et al., 2013).

The *Arabidopsis* genome contains four Dicer-like enzymes, of which only DCL1 is responsible for miRNA maturation while the others generate siRNAs (Unver et al., 2009). The miRNA-miRNA* duplex has 3' overhangs of two nucleotides on each strand. The plant miRNAs (but not animal miRNAs) then undergo methylation on 2'OH on each 3' terminal nucleotide by Hua Enhancer 1 (HEN1). The methylated duplex leaves the nucleus to enter cytoplasm with the assistance of the exportin family of transporters HASTY (Axtell et al., 2011) (Figure 2.7.2). The methylation is suggested to protect the miRNAs from degradation by uridyl-transferases from the 3' uridylation activity (Ren et al., 2014) and from the miRNA being used as primer for RNA polymerase for transcription of other genes (Guleria, 2011). Unlike uridylation, adenylation has been suggested recently to stabilize the duplex in both animal and plant miRNAs (Lu et al., 2009).

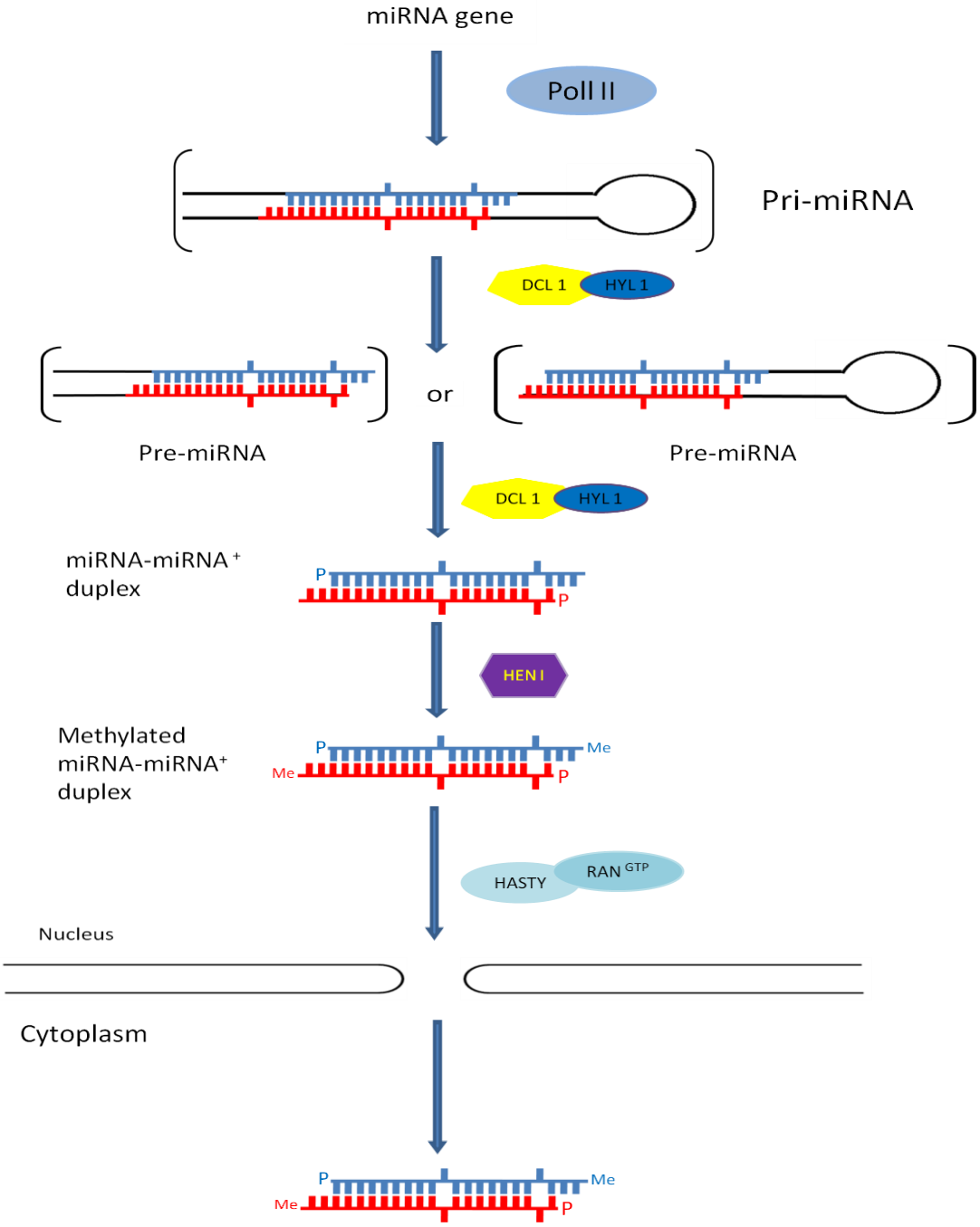

Figure 2.7.2 miRNA biogenesis in plants. Mature microRNAs are 21–24 nucleotides in length and processed in the nucleus from miRNA genes. The miRNA genes are transcribed by RNA polymerase II into primary-miRNAs (pri-miRNA), which then undergo 5' capping and 3' polyadenylation, some of miRNA genes locating in intron sequences. After transcription, the pri-miRNA is processed by DCL1, with the aid of HYL1 and other factors such as such as C2H2 zinc-finger protein SERRATE (SE) and nuclear CBC to shorter stem-loop structure called pre-miRNA with approximately 70

nucleotides in length. Pre-miRNA is continuously processed by DCL1 to a miRNA:miRNA* duplex with 5′ phosphates (P) and two-nucleotide 3′ overhangs. The miRNA:miRNA* duplex are then methylated (Me) by HEN1 and exported to the cytoplasm by HASTY with the aid of additional factors.

2.7.3 Loading of miRNA into the silencing complex

After being exported to the cytoplasm, the miRNA-miRNA* duplex is unwound by a helicase to release the mature miRNA (Figure 2.7.3) (Guleria, 2011). The miRNA mainly function through incorporation into the RNA-induced silencing complex (RISC). The RISC has a protein called Argonaute (AGO), which is a key component of the complex. The AGO proteins are highly conserved among eukaryotes and have three important domains, the PAZ, MID and PIWI, that are responsible for the function of target cleavage (Thieme et al., 2012). PAZ is an RNA-binding domain that binds to 3' end of miRNA, while MID binds to the 5' end of the target mRNA (Vaucheret, 2008, Tolia and Joshua-Tor, 2007). PIWI functions as RNaseH enzyme, cleaving the target mRNA at the position binding to 10^{th} and 11^{th} nucleotides of miRNAs by exonuclease activity (Peters and Meister, 2007, Rivas et al., 2005).

The number of members in the AGO family differs among different species. *Arabidopsis* and rice have contain 10 and 18 AGOs, respectively (Yu and Wang, 2010). Interestingly, studies in *Arabidopsis* showed that AGO1 has a role in mRNA cleavage, AGO7 and AGO10 bind to miR390 and miR165/166 respectively (Montgomery et al., 2008), while AGO4 and AGO6 take part in siRNA-mediated regulation of DNA methylation associated with gene silencing (Yu and Wang, 2010). Deep sequencing also uncovered that the process of miRNA binding to RISC was based on its 5' nucleotide; AGO1 of *Arabidopsis* binds to miRNA with 5' uridine; AGO2, AGO4, AGO6 and AGO9 prefer 5' adenine, while AGO5 associates with 5'cytosine (Thieme et al., 2012). It also appeared initially that only the miRNA strand of the duplex was loaded into RISC, with the miRNA* strand being degraded (Mourelatos et al., 2002). Czech et al. (2009), however, showed that the miRNA* was not always degraded, and could be incorporated with other AGOs to become stable and functional.

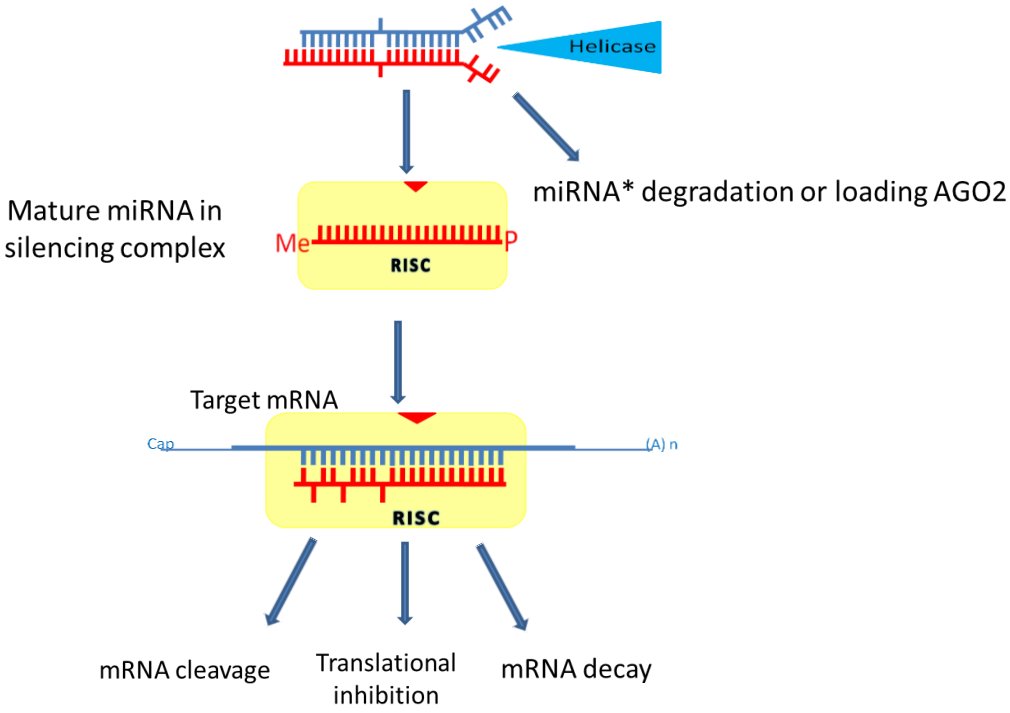

Figure 2.7.3 Mechanism of miRNAs in plants (adapted from Jones-Rhoades et al. (2006). In the cytoplasm, the miRNA:miRNA* duplex is unwounded by helicase to release mature miRNA. The miRNA* is degraded or loaded into AGO2 to another pathway. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC), containing AGO1. Target mRNA binding miRNA-RISC complex is capable for binding to target mRNA for cleavage by AGO1, translational prevention or degradation of mRNA depending on level of base pairing between miRNA and its target mRNA.

2.7.4 Turn-Over of miRNA

The miRNAs were thought to be stable sequences, however recent studies have discovered mechanisms that can accelerate the decay of miRNAs. The miRNA turn-over was shown to be crucial for proper growth as maintenance of normal development. In *Chlamydomonas reinhardtii*, MUT68 (a terminal nucleotidyltransferase) and RRP6 (ribosomal RNA processing protein 6 in 3'-5' exosome RNase complex) were found to take such a role, wherein MUT68 adds polyU to the 3' end of miRNAs, followed by cleavage by RRP6 (Ibrahim et al., 2010). In *Arabidopsis*, a family of small RNA degrading nucleases (SDNs), exoribonucleases that can specifically cleave single-

stranded mature miRNAs was reported, with their activity not affected by the 2'O methylation but inhibited by 3' oligouridylation (see above) (Ramachandran and Chen, 2008). The researchers also later showed *Arabidopsis* to encode HEN1 SUPPRESSOR1 (HESO1), a terminal nucleotidyltransferase which promotes 3' uridylation of miRNAs, but its activity is inhibited by 2'O methylation (Zhao et al., 2012). Therefore, SDN1 and HESO1 were supposed to cooperate in degradation of miRNAs (Rogers and Chen, 2013).

A recent study found another nucleotidyl transferase, UTP:RNA URIDYLYLTRANSFERASE (URT1), from *Arabidopsis* that can tail miRNAs at 3'end (Tu et al., 2015). The URT1 and HESO1 act in a different way to miRNAs, with a different 3'end and form tailing from the same miRNA (Tu et al., 2015). In addition, a study of larch (*Larix leptolepis*) found that nucleotide addition can take place at the 3'ends to promote the degradation of the miRNAs (Zhang et al., 2013). Targets of miRNA are also proposed to induce miRNA degradation by SDNs and methylated miRNA can be also degraded by unknown enzymes (Sanei and Chen, 2015) (Figure 2.7.4). Many animal miRNAs had been studied for their half-life that accelerated decay within 10 hours after production has been arrested (Rüegger and Großhans, 2012), however, there have been no studies reporting the decay rate of plant miRNAs.

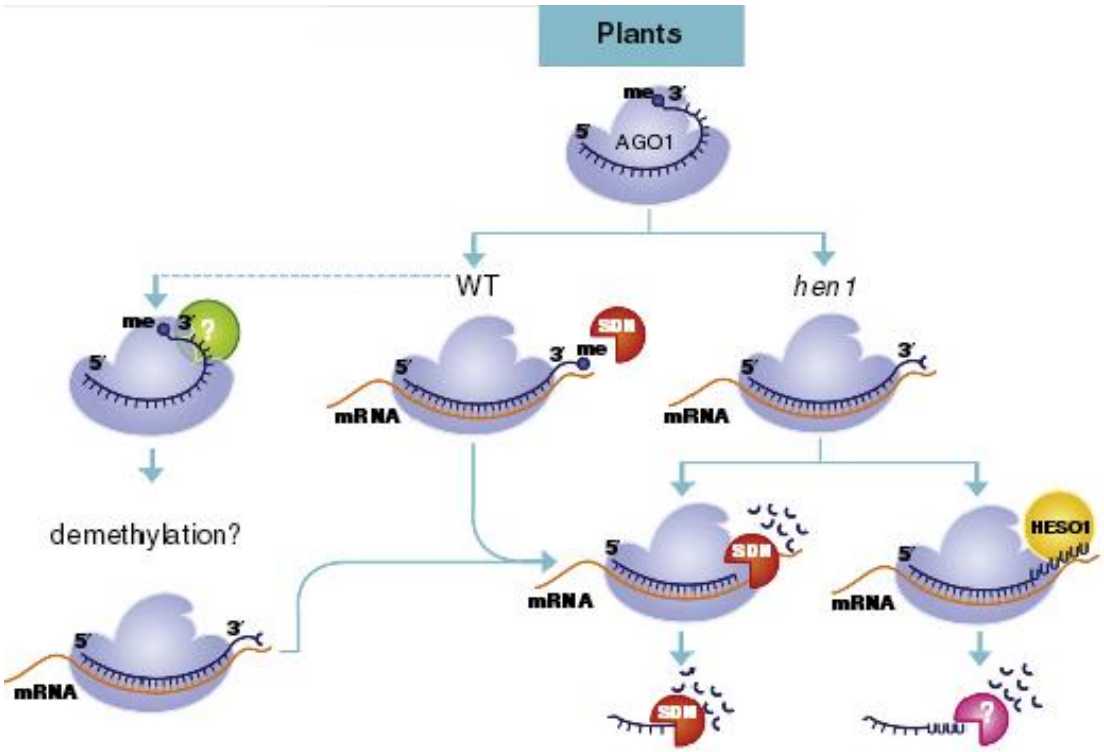

Figure 2.7.4 miRNA turnover (adapted from Sanei and Chen (2015). In the absence of HEN1 activity, unmethylated miRNA cannot be methylated, resulting in degradation by either 3' trimming using exonucleases SDNs or tailing with nucleotidyl transferase HESO1and URT1. In addition, methylated miRNA can be also degraded by an unknown enzyme.

2.8 Mechanism of regulation of gene expression by miRNAs

When the miRNA-loaded RISC binds to the target mRNA at the binding sites through sequence complementarity, AGO from the complex acts as an endonuclease on the mRNA by two main mechanisms: mRNA cleavage, or miRNA-direct translational inhibition (translational repression) (Budak and Akpinar, 2015).

2.8.1 mRNA cleavage

mRNA cleavage has been suggested to occur when the miRNAs complement their target mRNAs almost perfectly (Rhoades et al., 2002). The AGO of RISC cleaves the phosphodiester bonds in the mRNA, releasing its fragments. RISC is then liberated, to be incorporated into another miRNA and cleave further transcripts (Jones-Rhoades et al., 2006). For example, miR398 in *Arabidopsis* directly cleaves the mRNAs of CSD1 and CSD2 which encode Cu/Zn superoxide dismutases, enzymes in the redox regulation

pathways. Interestingly, the miR398 expression is decreased under oxidative stress, which allows CSD1 and CSD2 accumulation, resulting in stress tolerance (Sunkar et al., 2006). In barley, miR160 was reported to cleave its target ARF17, an auxin response transcription factor regulating cell elongation and division (Figure 2.8.1). Accumulation of barley miR160 down-regulated ARF17 expression under heat stress (Kruszka et al., 2014).

Figure 2.8.1 Diagram showing the slicing site of ARF17 binding hvu-miR160a (adapted from Kruszka et al. (2014). The arrow indicates the position of target cleavage validated by 5'RACE and the number of clones analysed.

2.8.2 Translational inhibition/repression

Translational inhibition/repression likely occurs in cases of imperfect base pairing between miRNAs and target mRNAs; however, there are differences in the activity of the miRNAs, with the rate of inhibition appearing to be related to the number binding sites of miRNA on mRNA. Several studies (Liu et al., 2005, Sen and Blau, 2005, Huang et al., 2011) have noted that miRNA-RISC is located at the cytoplasmic foci (P bodies), where mRNA can be stored or degraded. Binding of the target mRNA to miRNA-RISC at P body sites suggested that mRNA can be moved from translation to degradation. A different explanation was reported later (Guleria, 2011), that the miRNAs bind at the 5' UTR, ORF, or 3' UTR of the target mRNA and prevent the movement of ribosomes, leading to translational suppression. An example of this mechanism is found in *Arabidopsis*, wherein expression of miR399 was up-regulated under low phosphate. Its induction decreased the expression of its target, the UBC24 mRNA (which has a role in protein degradation) directly by binding to its 5'UTR and causing translational repression. Thus the level of UBC24 expression regulated Pi uptake in response to phosphate stress (Chiou et al., 2006).

2.9 Roles of miRNAs in an abiotic stress response to plants

miRNAs have been shown to be involved in the regulation of many plant processes such as development (Reinhart et al., 2002), flowering (Aukerman and Sakai, 2003), auxin signalling (Mallory et al., 2005), responses to abiotic (Sunkar and Zhu, 2004) and biotic stresses (Zhang et al., 2006), or regulation of their own biogenesis (Dugas and Bartel, 2004). The ability of plants to respond *in situ* to abiotic stresses such as salinity, drought, heavy metals, temperature extremes or nutrient deprivation is critical for their survival, as unlike animals, they are immobile. A number of miRNAs have been shown in the last few years to have key roles in regulation of plant responses to environmental factors; these are summarised below.

2.9.1 Salt stress

Soil salinity is one of the most common stress factors worldwide and severely affects plant growth and crop yield, as discussed earlier. In *Arabidopsis*, expression of a number of miRNAs (miR396, miR397, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, miR158, miR169) was found up-regulated during salt stress by microarray analysis (Liu et al., 2008), while only miR398 was down-regulated under qPCR assay (Jagadeeswaran et al., 2009). Induction of miR397 was found to down-regulate its targets, laccase-like protein (LAC) and a regulatory subunit of casein kinase (CKB3), by direct cleavage of their mRNA (Zhu et al., 2007). The targets of miR398 are Cu/Zn superoxide dismutases CSD1 and CSD2, increases in their expression levels being related to down-regulation of miR398 (Sunkar et al., 2006). In rice, miR169g and miR169n (from the miR169 family), as detected by qPCR, increased dramatically under salt (Zhao et al., 2009) and were found to regulate NF-YA5 (a CCAAT-box binding transcription factor) by direct cleavage of its mRNA (Li et al., 2008). MiR169 was also induced upon salt treatment in *Arabidopsis* (Zhao et al., 2009). miR169 was, however, noted to be up-regulated in the short term (0-48h h) and then down-regulated in the long term (15 days) of salt stress while its target, NF-YA, raised the expression level in hours and the transcripts then dropped on day 15 of treatment (Luan et al., 2015). Another study using qPCR found 98 miRNAs from 27 families in maize root responded to salt stress. The targets of miR159a/b, miR164a/b/c/d and miR1661m were identified as transcription factors Myb and NAC and a homeodomain-leucine zipper protein (HD-ZIP) (Ding et al., 2009). A study using qPCR also found 8 families (miR159, miR160, miR167, miR174, miR399, miR408, miR1124, miR1133) to be up-regulated during salt stress in wheat (Lu

et al., 2011). Recent study showed 385 conserved and 68 novel miRNAs have responded differently to *Medicago sativa* and *Medicago truncatula* under salt stress (Long et al., 2015).

2.9.2 Dehydration stress

An adequate water supply is needed for proper plant growth and development, and over- or under-supply can cause significant damage, as discussed earlier. Many researches have demonstrated crucial roles of miRNAs in responding to water stress. In *Arabidopsis*, microarray analysis showed that some miRNAs (miR157, miR167, miR168, miR171, miR408, miR393, miR396) had increase in expression levels during drought (Liu et al., 2008), while only miR169 was down-regulated (Li et al., 2008). The target of miR169 was found to be NFYA5 (a member of NFYA family), a transcription factor with a role in stress response, a decrease in miR169 expression leading to increase in NFYA5 transcript levels (Li et al., 2008). In rice, eight families (miR395, miR474, miR845, miR851, miR854, miR901, miR903, miR1125) showed significant up-regulation and 11 families (miR170, miR172, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088, miR1126) showed down-regulation using microarray analysis, confirmed by quantitative PCR (qPCR) (Zhou et al., 2010b). Another study using wild rice (*Oryza rufipogon*) showed 17 miRNAs were down-regulated significantly and 16 were up-regulated upon drought stress (Zhang et al., 2016). In *Populous trichocarpa*, all ten miRNA families detected via microarray to respond to drought stress (miR1446, miR1444, miR1447, miR1450, miR1711, miR482, miR530, miR827, miR1445, miR1448) showed a decrease in expression (Lu et al., 2008), while in *Medicago truncatula,* two families (miR398, miR408) were found by qPCR assay to be up-regulated under water stress conditions (Trindade et al., 2010). A study of Kantar et al. (2011) using qPCR analysis in wheat under drought treatment found 13 miRNA families responded to drought stress. Of these, eight families (miR1867, miR398, miR474, miR156, miR894, miR1432, miR1881, miR1450) showed an increase, four families (miR528, miR166, miR171, miR396) showed a decrease, and miR896 fluctuated. The targets of miR398, miR474, miR166, miR156, miR171 and miR396 were found to be genes involved in plant development and/or abiotic stress responses, i.e., copper superoxide dismutases, kinesin, HD-ZipIII, Squamosa-promoter binding protein (SBP), GRAS domain and growth regulating factor-like (GRL) transcription factor. Study of barley under drought treatment by qPCR also showed that miR156, miR166, miR171 and miR408 were induced (Kantar

et al., 2010), while miR164, miR166, miR167, miR168, miR169, miR172, miR319, miR396 and miR827 were down-regulated (Hackenberg et al., 2012). The contradictory data for miR166 in these studies need further investigation. In maize, microarray data showed 39 miRNAs responded to submergence, and the bioinformatically predicted targets of 38 of these appeared to be involved in signal transduction, cell defense, differentiation and carbohydrate metabolism (Zhang et al., 2008).

2.9.3 Cold stress

Low temperature extremes such as chilling and freezing cause severe stress in unadapted plants, as discussed earlier. In *Arabidopsis*, microarray data showed several cold-responsive miRNAs to be up-regulated (miR165/166, miR169, miR172, miR393, miR396, miR397, miR402, miR408) (Zhou et al., 2008) while miR398a levels decreased (Sunkar and Zhu, 2004). MiR172 was found to regulate the transcription factor AP2 (APETALA) by translational attenuation (Zhou et al., 2008). In *P. trichocarpa*, expression of 15 miRNAs assessed through microarray was found to be up-regulated during cold stress, while miR156g-j, miR475a, b and miR476a were down-regulated (Lu et al., 2008). In rice, also using microarray analysis, 18 miRNA families responded to cold, most of them decreasing in expression, except miRNA171 members which showed varying expression (Lv et al., 2010). Study of Thiebaut et al. (2012) on sugarcane by qPCR noted that miR319 was induced but decreased after 48h of cold treatment, and that miR319 regulated its targets, the Myb and TCP transcription factors, by direct cleavage.

2.9.4 Hypoxia

Hypoxia can affect mitochondrial respiration and change the respiratory mechanism in plants from being aerobic to anaerobic. Forty-six miRNAs from 19 families in *Arabidopsis* showed an altered expression during hypoxia (Moldovan et al., 2010). In maize, the microarray showed varied expression patterns; while miR167, miR166, miR171 and miR396 were induced at early stage, miR159, miR395, miR474 and miR528 were down-regulated during early stage but increased after long term exposure (Zhang et al., 2008).

2.9.5 Oxidative stress

Excessive accumulation of ROS can occur under diverse stress conditions and can damage cells, as discussed earlier. Oxidative stress can be reduced by superoxide

dismutases (SOD), a group of peroxidase and catalase enzymes which can detoxify superoxide radicals into H_2O_2 (Sunkar, 2010). In *Arabidopsis*, miR398 was found to be oxidative stress responsive and could cleave CSD1 and CSD2 transcripts, resulting in oxidative tolerance (Sunkar et al., 2006).

2.9.6 Abscisic Acid (ABA) stimulation

The plant stress hormone ABA plays a key role in certain abiotic response signalling networks. In *Arabidopsis*, Sunkar and Zhu (2004) found miR393 expression to be up-regulated during ABA stress, and this miRNA targeted mRNA that encoded the TRANSPORT INHIBITOR RESPONSE 1 (TIR1). TIR1 is involved in proteolysis of Auxin/Indole 3-Acetic Acid (AUX/IAA), which has a role in plant growth and development (Dharmasiri and Estelle, 2002). Accumulation of miR393 cleaves *TIR1* mRNA or prevents its translation, resulting in negative effects on auxin signaling and plant development. MiR417 was found by qPCR to be up-regulated at early stage upon ABA treatment but down-regulated later, and led to reduce seed germination and seedling survival (Jung and Kang, 2007). MiR159 was also induced by ABA stress, and its accumulation increased the degradation of *MYB101* and *MYB33* transcripts that encode ABA-responsive positive regulators, thus diminishing ABA signaling (Reyes and Chua, 2007). A further understanding of these miRNAs and their targets is essential, as ABA is central to abiotic stress response pathways in plants.

2.9.7 Nutrient Stress

Plants absorb inorganic sulphate and transport it into root and leaf xylem cells where it is assimilated into cysteine, which then takes part in synthesis of proteins and other molecules (Rausch and Wachter, 2005). In *Arabidopsis*, miR395 was the first sulphate-responsive miRNA identified, and its targets found to be ATP SULPHURYLASES (APS) (involved in sulphate assimilation) (Jones-Rhoades and Bartel, 2004) and AST68 (a transporter that assists with sulphate translocation) (Allen et al., 2005). Over-expression of miR395 reduced *APS* expression and led to decrease in sulphate metabolism during sulphate deficiency (Jones-Rhoades and Bartel, 2004).

Inorganic phosphate (Pi) is one of the most important components in the structures of nucleic acids and cell membranes as well as in other biological functions. The targets of *Arabidopsis* miR399 were identified to be transcripts of the phosphate transporter

(PHO2) (Jones-Rhoades and Bartel, 2004) and ubiquitin conjugating enzyme (UBC24; with roles in phosphate homeostasis) (Sunkar and Zhu, 2004). MiR399 was up-regulated upon Pi starvation, its accumulation decreasing the ubiquitin/proteasome pathway that represses the transporter expression and increases Pi loading (Chiou et al., 2006). The transcription factors MYB and PHOSPHATE STARVATION RESPONSE 1 (PHR1) were involved in miR399 response to Pi deficiency; they share a MYB domain that binds to the GNATATNC cis-element of miR399 and up-regulates its expression (Chiou et al., 2006, Pant et al., 2008).

Copper, a trace element, is essential for key processes including photosynthesis and respiratory electron transport. In *Arabidopsis*, miR398 showed increased expression under copper starvation (Yamasaki et al., 2007), leading to negative regulation of *CSD1* and *CSD2* mRNAs. Burkhead et al. (2009) found that miR397, miR408 and miR857 were also up-regulated during copper starvation and decreased the expression of their targets, the mRNAs of plantacyanin and laccases, making copper available to other vital proteins.

The roles of miRNAs in plant development and responses to diverse environmental factors summarized above make a strong case for the need to study miRNA-regulated gene expression events in important crop species.

2.10 MiRNA in barley

Since the identification of miRNAs in *Arabidopsis* (Reinhart et al., 2002), a number of plant miRNAs have been identified and deposited in miRBase v21.0 (http://www. mirbase.org), which now contains more than 28,000 entries, belonging to *Arabidopsis* (427), rice (713), maize (321), sorghum (241) and *Brachypodium* (525).

2.10.1 miRNA in barley: the study so far

The study of barley (*Hordeum vulgare* L.) miRNAs is in its early stages compared to other species. The first large scale work on barley miRNAs was performed by Schreiber et al. (2011), who discovered up to 100 miRNAs through deep sequencing, however this work only investigated the leaf tissue. Lv et al. (2012) addressed this limitation by analysing miRNAs of barley from different tissues, i.e., roots, stems, leaves and spikes at different stages of development, leading to 126 conserved and 133 novel miRNAs.

However, our searches of this database found only 47 of these to be registered (http://www.mirbase.org; last accessed May 2016).

Currently, 71 mature miRNAs are registered as hvu-miRNA on miRBase (Release 21, accessed May 2016), while other 36 are predicted homologues of species such as rice, wheat or *Brachypodium*. Twenty six of the 71 barley miRNAs belong to 12 families. All are 20-24 nucleotides in length, released from stem-loop structures of about 70-300 nucleotides. Especially in miR168 and miR171 families, each stem-loop appears to have two mature miRNAs, located at the 5' and 3' of it, that complement to each other. Since barley genome sequence has become available in November 2012, Wu et al. (2014) have performed a large amount of work on the identification and characterization of conserved barley miRNAs *in silico* using the published draft genome assembly of barley cultivar Morex. 116 mature miRNA sequences were found in the barley genome assembly from 5,940 plant mature miRNAs in miRBase release 19. Of these, only 20 sequences were identified to 71 barley miRNAs deposited in miRBase. Most of sequences were located in intergenic or intronic regions. A total 610 genes were predicted as miRNA targets with various functions such as flowering, growth and response to environment stress in this study (Wu et al., 2014). Based on literature above (section 2.5) , barley appears to have 17 conserved miRNA families shared with other species such as rice, wheat, *Brachypodium*, *Arabidopsis*, *Populus*, maize and *Sorghum* (Table 2.5). However, our analysis found only nine of these in miRBase, the other eight being unregistered. The conserved miRNAs are well-supported to have important roles in response to biotic and abiotic stress (Kruszka et al., 2013). Schreiber et al. (2011) also described 44 other putative miRNAs in barley, not known to be expressed in other species and also lacking prior bioinformatic predictions. The candidate targets of these novel miRNAs are transcription factors or vital enzymes that suggest these miRNAs may also have roles in regulation of plant development.

The recent work of Kruszka et al. (2013) is the first major report investigating barley miRNA biogenesis as well as regulation of intron-derived miRNAs. Study of pri-miRNA of eight mature miRNAs (miR156g, miR159b, miR166n, miR168a, miR171e, miR397b, miR1120, miR1126) of barley showed that miR397b was generated from an intronless gene while miR156g and miR1126 were produced from introns of non-encoding MIR156g and MIR1126 genes. MiR168a had two different sequences in the same precursor, miR168a-5p and miR168a-3p, that were complementary to each other

and also expressed as mature miR168a. Interestingly, the target of miR168 was AGO1, showing the role of miR168 in negative regulation of miRNA biogenesis. The miR156g and miR1126 precursors were also present in introns. The MIR156g gene has six exons and five introns, with the first intron containing miR156g-miR156g* duplex, while the MIR1126 gene contains seven exons and six introns, the miR1126-miR1126* duplex being located in the third intron.

2.10.2 Barley miRNA response to abiotic stress

The role of miRNA in regulating responses of barley to the challenging and changing environments is the field of current interests. MiRNA capability of regulating response to drought stress in barley is supported by two studies. Kantar et al. (2010) noted that miR156, miR159, miR166, miR171 and miR408 were induced under drought stress and inversely correlated to their targets, SQUA promoter binding protein, MYB33, ARF, SCL6 and blue copper protein, respectively, that play role in plant development and architecture. Hackenberg et al. (2012) analysed the expression of miRNAs under the impact of TaDREB3 (a transcription factor) in transgenic barley. Over-expression of TaDREB3 resulted in survival of barley under drought or cold stress. Expression of a number of miRNA in both transgenic and non-transgenic plants was analysed, and miR156 had greatest induction in both plants, indicating it to be a strong candidate for drought tolerance. Another study of Hackenberg et al. (2015) found numerous drought-responsive miRNAs in barley cv. Golden Promise. Of 31 barley miRNAs detected, 13 showed significant decreases while one (hvu-miR5049b) displayed remarkable increase under drought stress. 20 out of 74 miRNAs orthologous to other species were down-regulated and one (gma-miR6300) was up-regulated, whereas two out of three novel barley miRNAs, hvu-MIRX34 and hvu-MIRX35, were reduced under drought condition and another hvu-MIRX33 only expressed upon normal condition. The expression of these miRNAs is also not consistent across different barley tissues, e.g., hvu-miR166a up-regulated in leaves but down-regulated in roots, hvu-miR168-5p up-regulated in leaves but unchanged in roots or hvu-MIRX35 only expressed in leaves but not in roots (Hackenberg et al., 2015).

A continuous study on the response to heat stress at level of mature and precursor miRNAs of four barley miRNAs (hvu-miR166, hvu-miR167, hvu-miR160 and hvu-miR5175) showed that all four miRNAs were increased under heat stress at both levels.

miR166 and miR167 were generated from intronless genes while miR160 was encode from the second intron of the gene containing 3 introns, miR5175 was located at the intron 10 of the *RNA polymerase II phosphatase-like/MIR5175a* gene (Kruszka et al., 2014). Increase in expression of all four miRNAs at both level suggested strongly effects on transcriptional and post-transcriptional regulation of miRNA expression under heat stress. Expression level of eight selected target genes, PHAVOLUTA (PHV), REVOLUTA (REV) and HOX9 (targets of miR166), ARF17 and ARF13 (targets of miR160), ARF8 and NEK5 (targets of miR167) and ACC-like oxidase (target of miR5175), were detected by real time PCR, showing the noticeable decrease under heat stress. PHAVOLUTA (PHV) has a role in regulating auxiliary meristem initiation and leaf development (Reinhart et al., 2002) while HOX9 regulates embryogenesis (Nagasaki et al., 2007). The inversely correlation between miRNAs and their targets proved that they might function in various regulatory network to cope with environmental stress in barley (Figure 2.10.1).

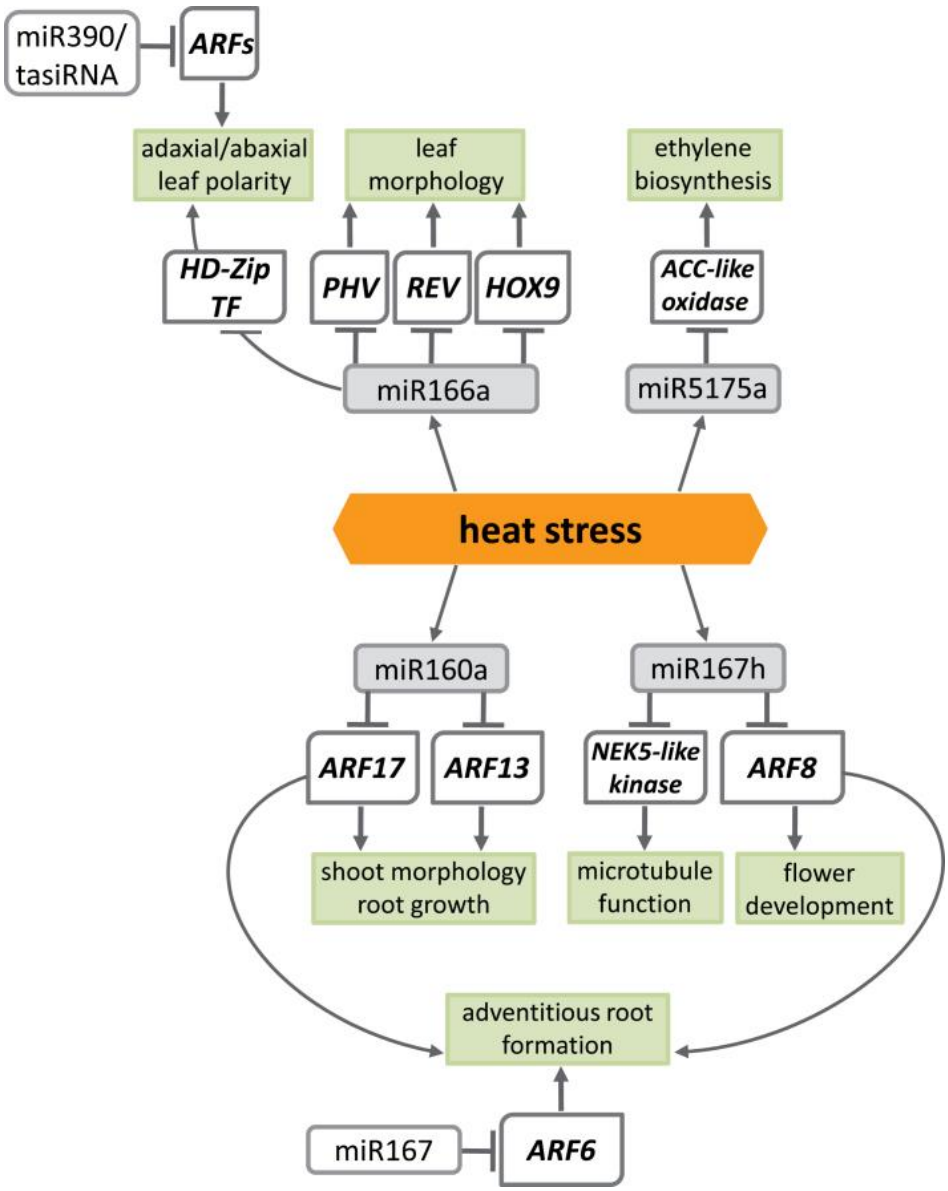

Figure 2.10.1 Correlation of miRNA-target response to heat stress in barley (adapted from Kruszka et al. (2014). Expression of miR160a, miR167h, miR166a and miR5175 increases under heat stress. These miRNAs inhibit the translation of their targets, subsequently affecting on barley growth. The arrows indicate positive regulation; the blunt-end lines indicate negative regulation.

miRNAs also take important role in barley response to nutrient deficiency. Study on miR399 and miR827 showed that their transcripts were significantly increased upon deficient Pi (Hackenberg et al., 2013b). The accumulation of miR399 and miR827 decreased the expression of their targets, PHO2 regulating phosphate uptake, allocation from root to shoot and SPX-domain-containing gene regulating other P-responsive genes,

respectively. miR399 has known to be Pi responsive miRNA in *Arabidopsis* (Chiou et al., 2006). This study confirmed the consistently specific role of miRNA among species. Expanded study from Hackenberg et al. (2013b) supported that hvu-miR1126 and hvu-miR5051 were also noticeable up-regulated in P deficient shoot besides hvu-miR399 and miR827. Other 7 up-regulated and 19 down-regulated conserved miRNAs as well as 3 significant increased novel miRNAs were also reported in this study. Intriguingly, hvu-miR168 and hvu-miR156 accounted for more than 95% of the annotated barley miRNAs in both control and stressed shoots, suggesting they do not function in regulating P status.

In 2013, Ozhuner and colleagues also reported a numerous miRNAs response to boron stress in barley. These authors found 31 known and 3 new miRNAs which 25 of them were boron responsive, however some miRNAs only expressed in tissue specific manner, e.g., miR156c and miR319a highly detecting in root while miR408 only expressing in leaf. Expression level was also different in specific tissues. In root tissue, miR165, miR2004 and miR5051 were increased while miR444b and miR2024a were decreased. In addition, miR156, miR169c, miR171, miR444a/c and miR2023a were up-regulated whereas miR156d, miR397, miR408, miR1121, miR2014, miR5049, miR5141 and miR5180 were down-regulated in leaf tissue (Ozhuner et al., 2013). Moreover, 934 barley transcripts were also predicted as targets of these miRNAs. miRNA408 was also proposed to have critical function in response to boron stress due to its striking expression in this study. Beside the known targets of miRNA408, Cu-binding domain containing chemocyanin and blue copper protein (Kantar et al., 2010), new predicted targets, heterotrimeric G protein (α) subunit and ATPase family gene 1 (AFG1), play important roles in signal transduction pathway (Ando et al., 2000, Bussemer et al., 2009, Buchanan-Wollaston et al., 2005) and a decreased expression of α subunit gene causing abnormal morphology in rice (Fujisawa et al., 1999).

miRNAs may also indirectly regulate other miRNA. A study showed that over-expression of miR171 decreased its target, SCL (scarecrow-like) transcription factor, affecting shoot development and flowering timing, and intriguingly, it could activate miR156 regulation (Curaba et al., 2013). The studies supported previous results (Curaba et al., 2012) that barley miRNAs may regulate early development of seed by targeting mRNAs involved in cell differentiation, energy mobilization, signalling pathways and defence responses to biotic and abiotic stresses. Beside barley, the role of miRNA in regulating vernalisation has been also studied in *Arabidopsis* (Oh et al., 2007) and peach

(*Prunus persica*) (Barakat et al., 2012). Together with the strong evidence of roles of siRNA in flowering regulation (Groszmann et al., 2011), these small RNAs seem to have significant roles in hastening vernalisation in plants.

The above reports thus contribute highly significant information on miRNA-mediated regulation of barley growth and development, especially in response to abiotic stress.

2.11 Some common putative targets of barley miRNAs involved in response to abiotic stresses

2.11.1 Squamosa (SQUA) promoter-binding protein

This transcription factor family facilitates flowering in plants (Xie et al., 2006). The proteins contain a highly conserved DNA-binding domain, a zinc finger motif with two binding sites (Cys-Cys-His-Cys and Cys-Cys-Cys-His). The transcript of this protein is the target of hvu-miR156 (Dryanova et al., 2008). Interestingly, a Squamosa promoter binding protein like 7 (SPL7) was detected as a regulator under copper deficiency in *Arabidopsis*. Unlike other Squamosa members, the DNA-binding domain of SPL7 has a C4 zinc finger motif that binds to the GTAC motif in the promoter region of miR398 gene and activates its transcription (Yamasaki et al., 2009).

2.11.2 MYB transcription factors

MYB proteins contain a highly conserved DNA-binding 'MYB domain' (Rubio et al., 2001) and have diverse functions in plants including developmental and metabolic processes, cell fate as well as biotic and abiotic stress regulation (Dubos et al., 2010). The transcript encoding a MYB33 transcription factor was identified as the target of hvu-miR159 (Dryanova et al., 2008). Study of 60 MYB genes from wheat (Zhang et al., 2012) showed that they responded to different stresses, 20 of these responded to multiple stresses, and 15 of these were regulated by ABA. Thus investigation of gene regulation by miRNAs under stresses is an important direction for wheat production.

2.11.3 GRAS family transcription factors

These factors regulate gene transcriptions and signal transductions during plant development (Bolle, 2004). The GRAS genes are reported from many species including *Arabidopsis*, rice, *Medicago truncatula* and *Lotus japonicas.* The GRAS proteins contain 400-700 amino acids forming motifs in the C-terminal such as the leucine heptad repeat I (LHR I) and repeat II (LHR II), VHIID and SAW, and are classified into eight groups (Hirsch and Oldroyd, 2009). The transcripts encoding GRAS family are targets of hvu-miR171 (Dryanova et al., 2008).

2.11.4 Homeodomain leucine zipper (HD-Zip) proteins

HD-Zip proteins have a homeo-domain (HD) and a leucine zipper motif (Zip) (Lee and Chun, 1998), and are divided into four subclasses according to the structures, unique domains and functions. HD-Zip I responds to abiotic stresses such as drought and light, HD-Zip II has roles in phototropism and auxin response, HD-Zip III in morphogenesis, and HD-Zip IV in processes such as epidermal fate, trichome formation and anthocyanin assimilation (Elhiti and Stasolla, 2009). The transcripts encoding this family are targets of hvu-miR166 (Hackenberg et al., 2012); it cleaves its targets directly, resulting in organ formation and regulation of the abiotic stress response.

2.11.5 CCAAT-binding transcription factor (NF-Y, CBF or HAP)

The CCCAAT-binding transcription factor is also known as nuclear factor Y (NF-Y), or CBF or HAP. The NF-Y family has roles in abiotic stress responses. NF-YB was reported to have a role in drought tolerance (Nelson et al., 2007), while NFYA5 controls stomatal aperture, its expression being induced by drought and ABA treatment (Li et al., 2008). NF-YA was also found to respond to salt stress (Zhao et al., 2009). However, the roles of many members of the family remain unclear. An NF-Y protein is made of three subunits (NF-YA, NF-YB and NF-YC), each of which is encoded by around 10 genes (Nelson et al., 2007). An mRNA encoding NF-YA is the target of hvu-miR169 (Dryanova et al., 2008). Induction of miR169 results in the cleavage of NF-YA, thus regulating the transcription of a number of downstream genes (Zhao et al., 2009).

2.11.6 Ubiquitination

The process of ubiquitination of proteins has important roles in response to abiotic stresses, such as phosphate starvation (Sunkar and Zhu, 2004) and salt and drought (Zhou et al., 2010a). Ubiquitination systems contain three enzymes; the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3), and the mRNAs encoding these are targets of hvu-miR399 (Dryanova et al., 2008).

2.11.7 Laccases

These copper-containing enzymes participate in lignin synthesis, with lignin having functions in water transport restriction. Laccases have diverse functions in plant development (Cai et al., 2006) and responses to biotic stresses such as pathogens and abiotic stresses such as salinity and copper homeostasis (Sunkar, 2010). The transcripts encoding laccase 2, 4 and 17 are targets of hvu-miR397 (Sunkar and Zhu, 2004), with the miRNA directly cleaving its targets, leading to tolerance to salt stress (Zhu et al., 2007).

2.11.8 Auxin response factor (ARF)

Auxin response factors (ARF) are transcription factors that regulate auxin gene expressions by binding to TGTCTC auxin response elements in the promoters of these genes (Tiwari et al., 2003). Auxin genes have role in cell division and elongation for plant growth and development (Woodward and Bartel, 2005). These proteins contain DNA-binding domain (DBD) and auxin/indole-3-acetic acid (AUX/IAA) domain in N-terminal and C-terminal region, respectively (Li et al., 2006). These also have middle region (MR) functioning either activation or repression domain (Ulmasov et al., 1999). Therefore, ARF can function as transcriptional activators or transcriptional repressors. The transcripts encoding these proteins are targets of miR160 and miR167 (Kruszka et al., 2014). The miR160 and miR167 regulate auxin gene expression by direct cleavage of ARF; these miRNAs, however, have yet registered in miRBase as barley miRNAs.

Part II MicroRNA in bacteria

2.12 Overview of small RNA in bacteria

The bacterial small noncoding RNAs have similar function to microRNA and siRNA in eukaryotes; however, they differ in their biogenesis and mechanism (Table 2.12.1**).** The concept of small non-coding RNA (sRNA) was first described for *E. coli* from 6S RNA in 1967 (Hindley, 1967) but did not receive public attention until recent when important role of small non-coding RNAs in post-transcriptional regulation in both eukaryotes and prokaryotes has been realised. In bacteria, the regulation of sRNA at post-transcriptional level was first demonstrated by finding of RNA I in plasmid ColE1 (Luan et al., 2015) and IS10 in the transposon TN10 (Simons and Kleckner, 1983). Prokaryotic non-coding RNAs usually contain 50-500 nucleotides in length (Gottesman and Storz, 2011) that are capable of folding stable stem-loop (Gottesman, 2005), which help maintaining the stability of the small RNAs (Massé et al., 2003, Vogel et al., 2003). Most of non-coding RNAs are involved in regulation of gene expression by base-pairing with mRNA, affecting on translation or stabilization of mRNA (Gottesman, 2005).

More than 100 small regulatory RNAs have been investigated in bacteria within the last 30 years (Murina and Nikulin, 2015). Most of them, 80 to 100 sRNAs belonged to *E. coli*, the most studied bacterium (Livny and Waldor, 2007, Altuvia, 2007), which is equal to around 1-2% of known protein-coding genes (Gottesman, 2005). sRNAs have also been identified and studied in several bacteria, e.g., *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Streptococcus pyogenes*, *Clostridium perfringens* and *Staphylococcus aureus* (Papenfort and Vanderpool, 2015). In the early 2000s, bioinformatics approaches have been employed to predict the presence of sRNAs in *E. coli* (Chen et al., 2002, Rivas et al., 2001). Since the development of DNA sequencing technology, numerous sRNAs have been detected in whole genome sequences using next generation sequencing (NGS) processes. In 2008, a program, SIPHT (sRNA identification protocol using high-throughput technologies), was developed to detect sRNAs obtained from bacteria. A collection of 932 bacterial replicons in the NCBI database was retrieved and analysed by using SIPHT software, yielding more than 45,000 novel sRNA candidates and 60% of previously identified sRNAs from *E. coli*, *V. cholera*, *P. aeruginosa*, *B. subtilis*, *L. innocua*, *P. marinus*, *S. typhimurium*, *S. aureus* and *S. meliloti* (Livny et al., 2008). The SIPHT software was also used to investigate sRNAs in

the genus *Rickettsia*, Gram-negative pathogenic bacteria causing spotted fever and typhus (Gillespie et al., 2007). Over 1,700 sRNAs were predicted in the intergenic regions of 16 different strains presenting 13 species in *Rickettsia* communities (Schroeder et al., 2015).

Most studies have been focused on bacteria that cause diseases in humans and animals. Nine putative sRNAs were identified by screening cDNA libraries of low-molecular weight RNA in *Mycobacterium tuberculosis*, the most prominent bacterial pathogens. These sRNAs were located within open reading frame or from intergenic regions that showed differential expression under various stress conditions and associated with genes encoding enzymes involved in lipid metabolic pathway (Arnvig and Young, 2009). Recent review showed more than thousand sRNAs were also identified in different species of streptococci by different bioinformatics prediction approaches (Patenge et al., 2015). The sRNA was also detected in the genus *Vibrionaceae*, marine bacteria causing infections on human as well as fish and marine invertebrates (Nguyen and Jacq, 2014). A study on marine *Synechococcus* revealed the presence of several known and 32 novel sRNAs using microarray profiling, showing different responses to various conditions. Among them, six sRNAs showed differential expression to cold stress, six sRNAs response to high light while another two sRNAs changed their accumulation to ion limitation (Gierga et al., 2012).

Table 2.12.1 Comparison of miRNA in plant and small RNA in bacteria: biogenesis and mechanism.

Features	**Plant**	**Bacteria**
Length	18-22 nucleotides	50-500 nucleotides
Stem-loop structure	Yes	Yes
Location in genome	Predominantly intergenic regions	Intergenic regions
Origin	MiRNA genes	Independent genes
Process take place	In nucleus and cytoplasm	In cytoplasm
Biogenesis	miRNA precursor is processed twice by DCL1 in nucleus miRNA:miRNA* is formed in nucleus Methylation at 3'end by HEN1	Transcription of small RNA is terminated by rho-independent transcription terminator
Incorporation	RISC	Hfq
Exonuclease	RNaseH	RNaseE
Turnover	SDNs, HESO1, URT1	RNaseE, degradosome
Mechanism	mRNA cleavage translation inhibition	Translation inhibition Translation activation

2.13 Biogenesis of small RNAs (sRNAs) in bacteria

2.13.1 Synthesis of small RNAs

The sRNAs are encoded in independent genes or generated together with mRNAs from mRNA leaders or trailers (Vogel et al., 2003). The process will be terminated by rho-independent transcription terminator (Gottesman, 2005). The non-coding RNAs were observed to be located in intergenic regions (not overlap coding regions) and remain conserved in related bacteria (Rivas et al., 2001).

Unlike eukaryotes, transcription and translation processes occur simultaneously in prokaryotic cells cytoplasm due to the lack of nuclear membrane. Therefore, mediation of translation can terminate the transcription of bacteria (Murina and Nikulin, 2015).

2.13.2 Binding to Hfq protein

After generation, sRNAs bind to their mRNA targets. If the sRNAs are perfectly complementary to their targets, they can regulate their targets without Hfq assistance (Nielsen et al., 2009), however Hfq protein is essential for acceleration of duplex formation between 'imperfectly complementary' RNAs (Kawamoto et al., 2006). Hfq, a homohexametric ring protein, is closely related to Sm and Sm-like proteins in eukaryotic RNA splicing (Sauter et al., 2003, Schumacher et al., 2002). Hfq has a similar function like RISC, and strongly interacts with small RNAs and target mRNAs at AU-rich regions, stimulating interaction between small RNAs and target mRNAs (Valentin-Hansen et al., 2004, Moller et al., 2002). A study on the interaction between Hfq and target mRNA showed that Hfq can alter the folding of mRNA subjected to binding to small RNAs (Geissmann and Touati, 2004). The small RNAs usually bind to the 5' end of mRNA transcripts, mostly at ribosome-binding site and/or starting codons (Gottesman, 2005). In addition, Hfq is also known to protect sRNAs from degradation by RNase E (Moll et al., 2003). Hfq protein has three RNA-binding sites forming two main binding regions: proximal side, a U-rich RNA binding site, and distal side, a poly(A) biding site (Murina and Nikulin, 2015) (Figure 2.13.1).

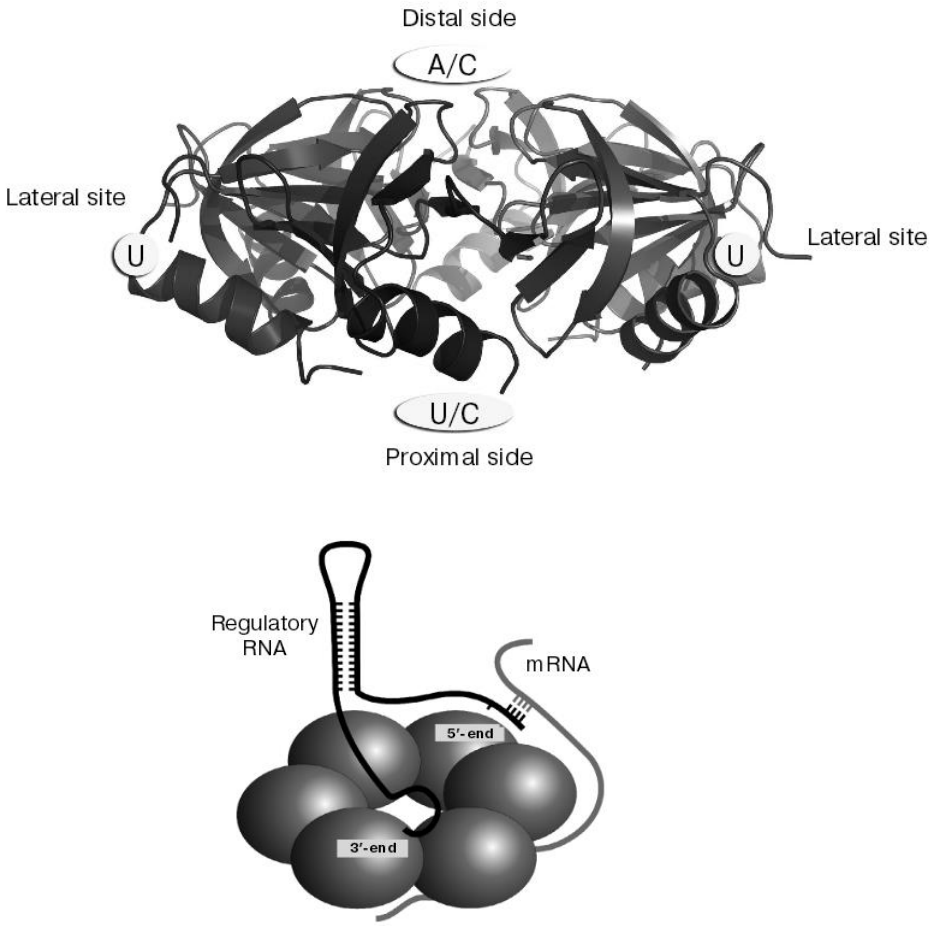

Figure 2.13.1 Structure of Hfq protein (I) and model for interaction of sRNA on the Hfq protein surface (II) (adapted from Murina and Nikulin (2015). (I) Arrangement of three RNA-binding sites on Hfq surface, Hfq recognized U-rich sequence at proximal side and poly (A) at distal side. It also has ability to bind to C-rich sequence in both RNA-interaction sites. (II) Interaction of sRNA on Hfq surface, 3'end of sRNA bind to central pore of Hfq proximal side while mRNA interacts with distal side. The sRMA-mRNA duplex forms at 5'end of sRNA in the lateral side.

2.13.3 Recruiting of RNase E

In *E. coli*, the Hfq protein also has a role in recruiting RNase E, an endoribonuclease, which inhibits translation or triggers RNA degradation (Morita and Aiba, 2011, Prévost et al., 2011). The enzyme is composed of two specific domains: the N-terminal domain containing site-specific endonuclease activity, and the C-terminal

domain, having a scaffold for the degradosome complex of polynucleotide phosphorylase, RNA helicase and metabolic protein enolase (Kido et al., 1996, McDowall and Cohen, 1996, Callaghan et al., 2004, Carpousis, 2002). The enzyme has a specific interaction with the AU-rich regions of single-stranded RNA (Carpousis, 2002). It has been shown that the efficiency and specificity for the cleavage site of the enzyme is influenced by the stem-loop structure, membrane binding and phosphorylation status at the 5' terminus of RNA substrates (Kime et al., 2010, McDowall et al., 1995, Celesnik et al., 2007), however this type of enzyme differs among species, especially between Gram-positive and Gram-negative bacteria with significant differences in degradation machineries (Condon and Putzer, 2002). In Gram-positive bacteria, RNase Y was found to replace RNase E of mRNA turnover in *Bacillus subtilis* (Commichau et al., 2009), *Staphylococcus aureus* and *Streptococcus pyogenes* (Kaito et al., 2005).

A study of the turnover of small RNA showed that these small RNAs and their mRNA targets are degraded simultaneously and were dependent on the presence of RNase E and degradosome (Massé et al., 2003). Small RNA turnover also depended on base-paring with their targets. They are stable when the transcription is inhibited and unstable when the transcription is in process.

2.14 Mechanism of regulation of gene expression

Bacterial small RNAs show imperfect complementarity with their target mRNAs in the pairing regions of 8-9 nucleotides (Gottesman, 2005). Unlike eukaryotes, incorporation of small RNAs and their targets lead to cleavage or inhibition of mRNA translation. Bacterial small RNAs can mediate RNA translation negatively or positively (Murina and Nikulin, 2015) (Figure 2.14.1).

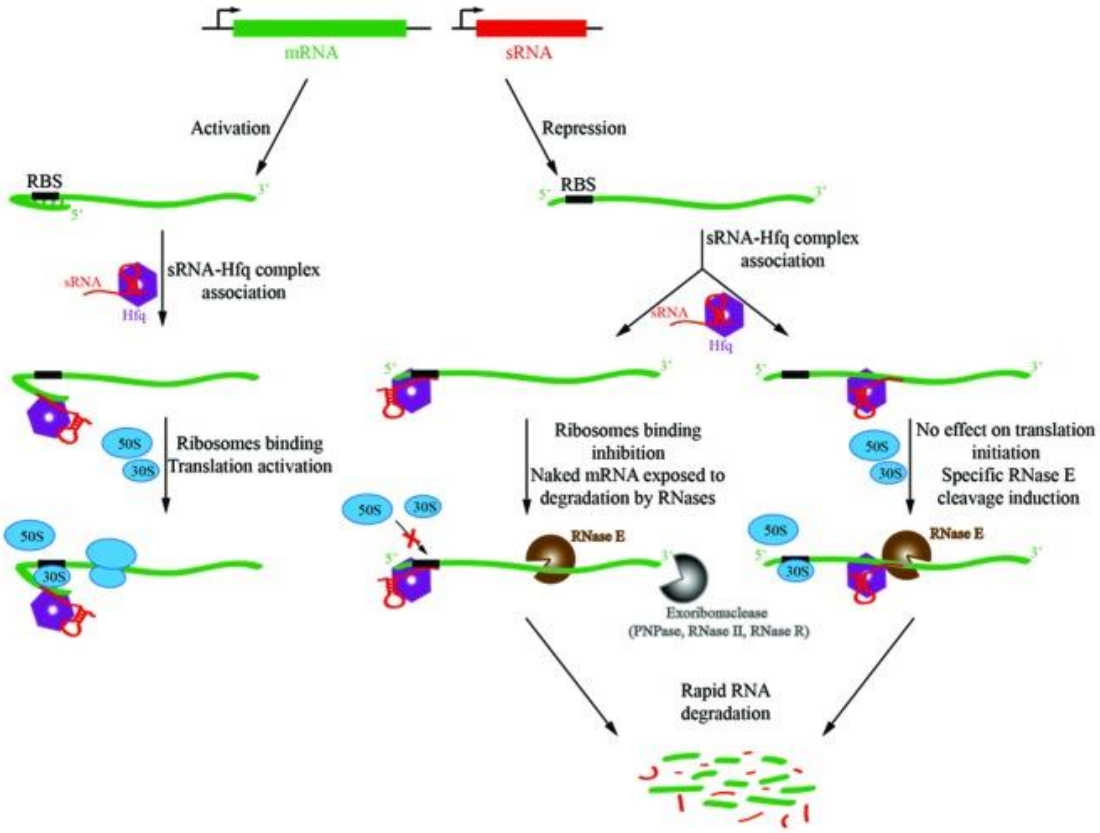

Figure 2.14.1 Mechanism of small RNA in bacteria (adapted from Bandyra and Luisi (2013). The sRNAs (red) is interacted with Hfq protein and bind to the target gene mRNA (green). When sRNA-Hfq complex binds to ribosome-binding site (RBS, or Shine Dalgarno sequence) or at any coding sequence, it inhibits ribosome associated to mRNA, preventing translational initiation and activating RNase E to cleave mRNA (right panel). Conversely, the sRNA-Hfq complex can bind to the 5'end of mRNA, unfold the hairpin structure at the 5'end and release the ribosome-binding site. Ribosome can then bind to the RBS and initiate translation (left panel).

2.14.1 Negative translation regulation

Negative translation regulation occurs in cases where sRNA binding takes place on the ribosome-binding site (RBS) on mRNA or at any coding sequence of mRNA.

At the ribosome-binding site, the sRNA forms a duplex with the mRNA, blocking the mRNA RBS. The ribosome cannot bind to mRNA to initiate translation, leading to a termination of the transcription by rho-dependent or rho-independent terminators. RNA can be cleaved by either RNase III or RNase E which cleaves the duplexes or single stranded regions, respectively and completely decayed by degradosome (Murina and

Nikulin, 2015). An example for this case is OxyS, a 109 nucleotide sequence, found to be accumulated in cells in response to an oxidative stress. The OxyS RNA binds to ribosome-binding site, inhibited translation of its targets *fhlA* (transcriptional activator) and *rpoS* (σ-factor of RNA polymerase) (Altuvia et al., 1998).

At the coding sequence, translation inhibition occur at elongation stage when small RNA form duplex with mRNA, preventing ribosome access for continuous process (Papenfort and Vanderpool, 2015). For example, a sRNA SR1 was reported to regulate arginine catabolism by modulating the expression of its target ahrC mRNA in *Bacillus subtilis* (Heidrich et al., 2007). SR1 inhibits ahrC mRNA translation by binding to a region 100 nucleotides downstream from the RBS, changing the structure.

2.14.2 Positive translation regulation

Positive translation regulation can occur when sRNAs interact with their mRNA targets at various positions: 5' UTR, coding sequence or 3' UTR of transcripts by diverse mechanisms: enhancement of ribosome accessibility to mRNAs, sequestration of RNase E recognition sequence, increase of mRNA stability or mimicry of targets (Papenfort and Vanderpool, 2015) (Table 2.14.1).

Table 2.14.1 List mechanism and sRNA in activation of target translation.

Organism	**sRNA**	**Role**	**Target**	**Reference**
Activation at the 5' UTR				
Staphylococcus aureus	RNAIII	Growth phase Cell density	Hla encoding α-toxin	Novick and Geisinger (2008)
Listeria monocytogenes	Rli27	Pathogenesis	Lmo0514	Quereda et al. (2014)
E. coli	DsrA RprA ArcZ	Stress response and virulence	rpoS	Mika and Hengge (2014)
	RyhB	Siderophore production and virulence	shiA	Porcheron et al. (2014)

Organism	sRNA	Role	Target	Reference
Vibrio harveyi	Qrr	Cell density	aphA	Rutherford et al. (2011)
Vibrio cholerae	Qrr	Biofilm formation	vca0939 encoding GGDEF protein	Zhao et al. (2013)
Stabilization of mRNA through sRNA binding at the 5' UTR				
Streptococcus	FasX	Pathogenesis	ska encoding streptokinase A	Ramirez-Peña et al. (2010)
Clostridium perfringens	VR-RNA	Metabolic function	colA encoding collagenase	Obana et al. (2013)
Escherichia *Salmonella* *Citrobacter* *Enterobacter* *Klebsiella* *Shigella*	RydC	Membrane stability	cfa encoding fatty acid synthase	Fröhlich et al. (2013)
Activation by base pairing within the mRNA-coding sequence				
E. coli *Salmonella*	SgrS	Glucose-phosphate stress	yigL encoding haloacid dehalogenase-like phosphatase	Fröhlich et al. (2013)
Activation by base pairing at the 3' end of target mRNAs				
E. coli	GadY	Glutamate-dependent acid resistance system	gadX-gadW	Opdyke et al. (2011)
Activation by molecular mimicry				
E. coli	GlmZ	Cell envelope synthesis	glmS	Papenfort and Vanderpool (2015)
Activation by changing RNA structure through Hfq				

Organism	sRNA	Role	Target	Reference
E. coli	RyhB	Ion physiology and colicin sensitivity	cirA	Salvail et al. (2013)
Activation by RNA 'sponges' for repressor sRNAs				
Salmonella	ChiX	Chitin utilization	ChiP	Figueroa-Bossi et al. (2009)

Some specific examples will be described in detail below:

An example of the enhancement of ribosome accessibility to mRNA is when an interaction takes place between rpo mRNA and sRNAs (DsrA, RprA and ArcZ). The rpo mRNA was found in an *E. coli* encoded σ-factor which has role in regulation of transcription initiation of genes response to stresses and virulence (Battesti et al., 2011). The mRNAs were reported as being conserved among Gram-negative bacteria (Soper and Woodson, 2008). In the absence of sRNA, rpoS folds into a hairpin structure at 5' UTR, hiding the ribosome-binding site (RBS) by forming a double-stranded secondary structure, preventing ribosome access. The interaction between sRNAs and rpoS relieves RBS, initiating the translation of a σ-factor (Mika and Hengge, 2014) (Figure 2.14.2).

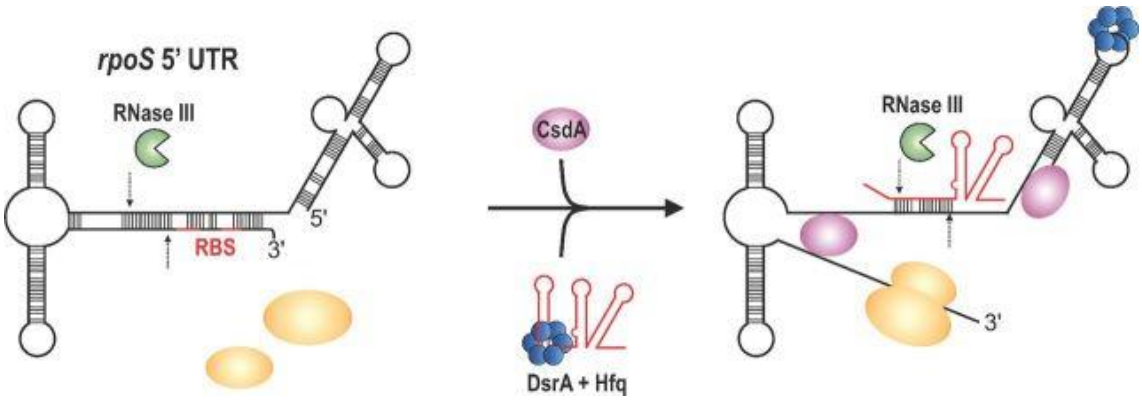

Figure 2.14.2 Activation of *rpoS* by DsrA (adapted from Papenfort and Vanderpool (2015). In *E. coli*, the translation of *rpoS* mRNA is inhibited by *rpoS* itself forming a complex secondary structure that blocks the RBS. This structure is also subjected the cleavage of RNase III. In the presence of DsrA, DrsA-Hfq association can bind to *rpoS* 5′ UTR, rearranging the *rpoS* structure for ribosome binding. Interaction with DsrA also alter RNase III cleavage site in the distal part of the stem-loop structure.

An example of interference with nucleolytic decay is SgrS, an 220 nucleotide long sRNA found in *E. coli* and other enteric bacteria (Horler and Vanderpool, 2009). SgrS was reported to regulate four targets which represses three targets: *ptsG, manXYZ* and *sopD* and activates one *yigL*, encoding a haloacid dehalogenase from bicistronic with the upstream pldB. SgrS binds to the 3' region of pldB coding sequence and stabilize the 'pldB-yigL' mRNA by sequestrating RNase E recognition sequence (Fröhlich et al., 2013) (Figure 2.14.3).

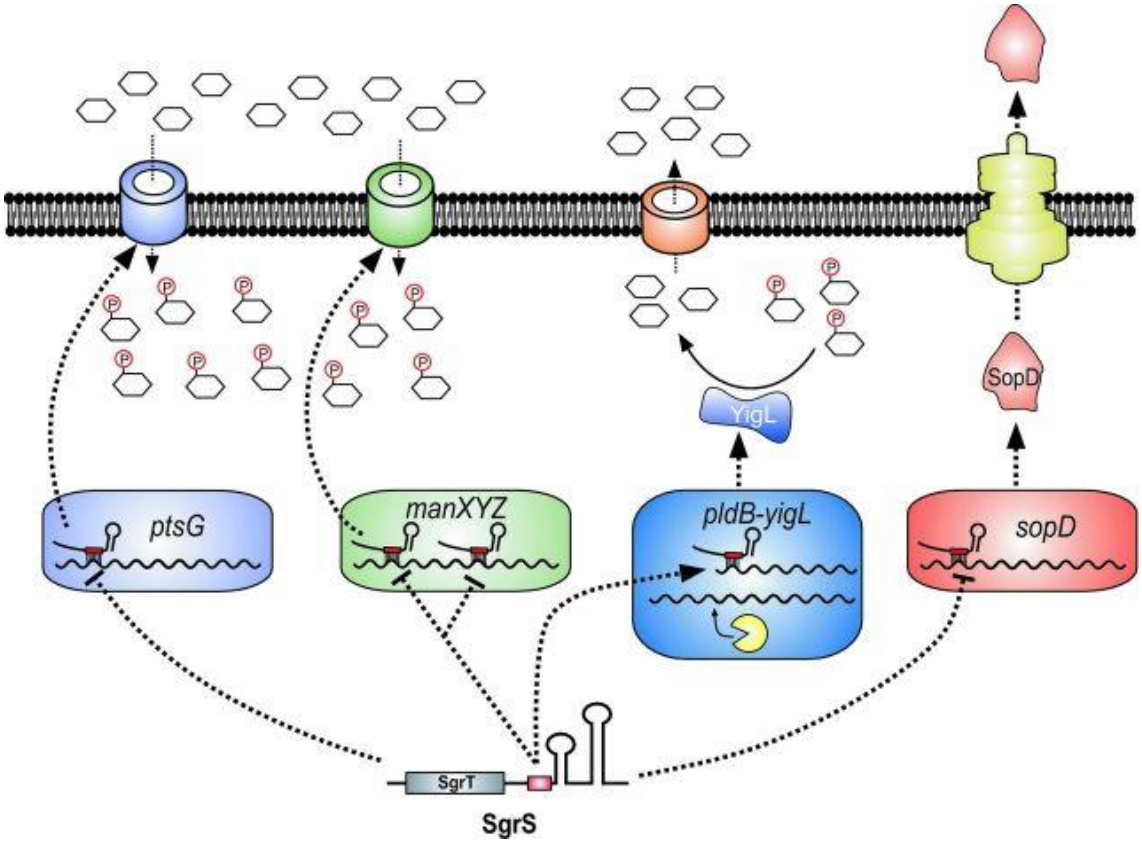

Figure 2.14.3 Mechanism of small RNA SgrS regulation in carbon metabolism and virulence (adapted from Papenfort and Vanderpool (2015). SgrS inhibit the translation of three mRNA targets, *ptsG, manXYZ* and *sopD*, and activates mRNA target, *yigL* mRNA. SgrS bind to 'pldB-yigL' bicistronic region and prevent mRNA degradation through RNase E. The activity reduces sugar uptake (via repression of *ptsG* and *manXYZ*) and increases the sugar efflux (via activation of *yigL*) for recovery from glucose-phosphate stress.

Increase of mRNA stability: GadY, 105 nucleotides in long, encoded in gadX-gadW intergenic region (Opdyke et al., 2004) involved in controlling glutamate-dependent acid resistance system in *E. coli* (Ma et al., 2002). GadY base-paired to gadX-gadW, creating double-stranded sequence within the complementary region of RNase III. The RNase III separated mRNA into gadX and gadW transcripts, which are more stable than the unprocessed transcript (Opdyke et al., 2011) (Figure 2.14.4).

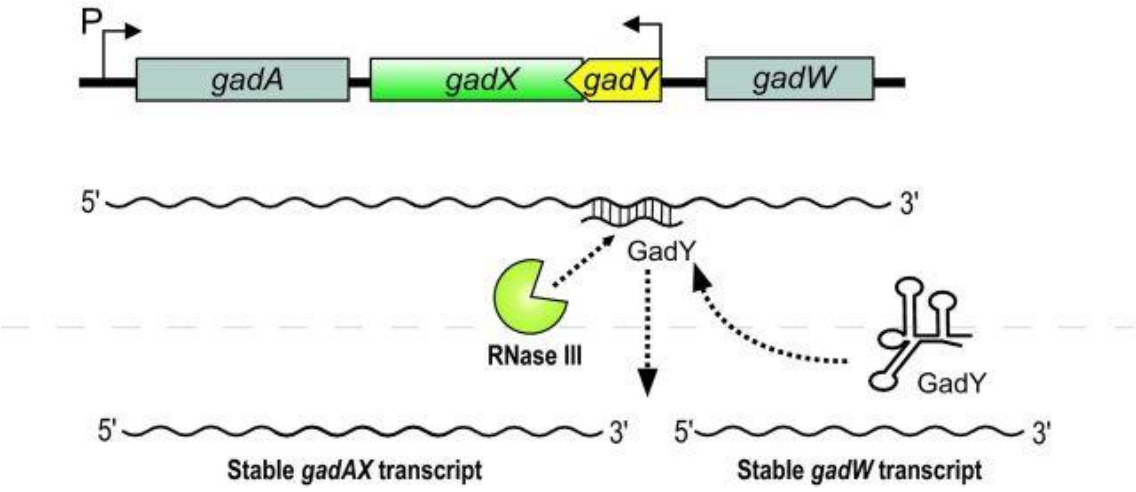

Figure 2.14.4 Target stabilization by GadY (adapted from Papenfort and Vanderpool (2015). The sRNA GadY interacts with the *gadX–gadW* intergenic region, creating a double-stranded sequence for RNase III processing. The activity yields individual *gadX* and *gadW* transcripts that are more stable than the full-length mRNA.

2.15 Role of bacterial sRNA

Like the role of miRNA in plants, bacterial small RNAs have been reported to play crucial roles in the adaptive response of bacteria to environmental factors such as cold stress (Sledjeski et al., 1996), iron deficiency (Wilderman et al., 2004) or high light stress (Gierga et al., 2012). In addition, these small RNAs also regulate genes controlling pathogenicity and virulence (Pfeiffer et al., 2007) and stimulate the gene expression response to population density (quorum sensing) (Lenz et al., 2004), however bacterial small RNAs present heterogeneity in size and structure and regulate their targets through different mechanisms (Hess and Marchfelder, 2012).

2.15.1 Iron metabolism

Iron is an essential metal for the growth and survival of bacteria as it is a cofactor of enzymes involving in essential processes such as photosynthesis, DNA metabolism, electron transport and respiration (Nguyen and Jacq, 2014, Repoila and Darfeuille, 2009). Levels of iron will be decreased in the presence of oxygen due to the catalysis of hydroxyl radicals and ion superoxides, causing oxidative stress (Touati, 2000). Maintenance of intracellular iron levels is an important physiological requirement for the avoidance of toxic effects. In bacteria, Fur (ferric uptake regulator) and sRNA RyhB (90 nucleotides) were reported to control iron uptake. Fur is a key transcriptional repressor (Hantke, 2001) that regulates the transcription of RyhB while sRNA RyhB controls the translation of iron-containing protein by binding to mRNA, inhibiting the translation and stimulating the degradation (Massé et al., 2007). At low levels of iron, Fur is active and represses the transcription initiation of RyhB, resulting in the expression of iron uptake genes. In addition, RyhB also inhibits the expression of genes involved in other metal metabolism and oxidative stress responses (Andrews et al., 2003). RyhBs have been detected in *E. coli* (Jacques et al., 2006), *Pseudomonas aeruginosa* (Wilderman et al., 2004) and *Vibrio cholera* (Davis et al., 2005).

2.15.2 Quorum-sensing (QS)

Quorum sensing is a bacterial cell-cell communication system that operates through the cells secreting signal molecules called autoinducers. When the accumulation of autoinducers is over a give threshold, the bacteria will respond to the signaling by adjusting their expression of genes (Waters and Bassler, 2005). A wide range of functions are regulated by the response of bacterial communities, including bioluminescence, virulence, biofilm formation, DNA exchange and sporulation (Bejerano-Sagie and Xavier, 2007). The quorum sensing system was first described in two marine bacteria, *Vibrio fischeri* and *Vibrio harveyi*, in 1979 (Nealson and Hastings, 1979). The system was also later found in *Vibrio cholera*. In this bacterium, the QS was found to prevent the production of virulence factors and the formation of a biofilm (Zhu et al., 2002, Hammer and Bassler, 2003). The 110 nucleotide RNA Qrr (quorum regulatory RNA) was first demonstrated as playing a role in QS by binding to luxR or hapR, transcriptional regulator of *V. harveyi* and *V. cholera*, respectively. LuxR and HapR bind to promoters of QS target genes and activate or repress their expressions. Therefore, Qrr indirectly regulate gene

expression in QS by blocking mRNA translation of luxR/hapR. Four Qrr1-4 were found in *V. cholerae* while five Qrr1-5 were detected in *V. harveyi* (Lenz et al., 2004, Tu and Bassler, 2007) (Figure 2.15.1).

In *V. cholerae*, the VarS-VarA (Virulence Associated Regulator) two-component system was identified as being regulators in QS. The VarS-VarA proteins act as kinase that regulates three small RNAs (CsrB, CsrC and CsrD) that are known to control the activity of CsrA. In turn, these RNAs indirectly modulate the expression of Qrr (Lenz et al., 2005) (Figure 2.15.1).

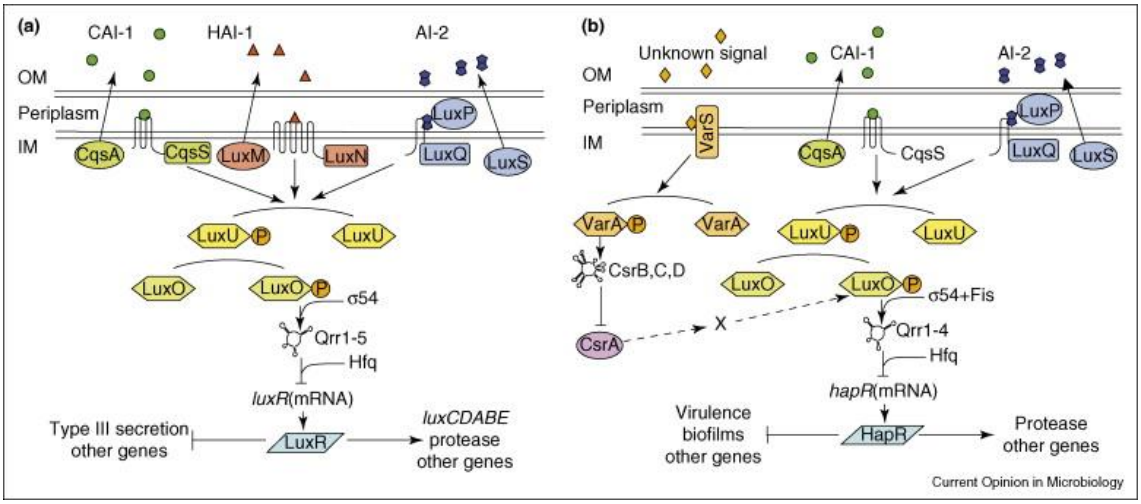

Figure 2.15.1 Quorum sensing (QS) systems of *Vibrio harveyi* (**a**) and *Vibrio cholerae* (**b**) (adapted from Bejerano-Sagie and Xavier (2007). **(a)** The *V. harveyi* QS consists of three autoinducers (CAI-1, HAI-1 and AI-2) that function in parallel. CAI-1 (green circles) is synthesized by the CqsA enzyme and binds to CqsS. HAI-1 (red triangles) is generated by LuxM and interacts with LuxN while AI-2 (blue double pentagons) is produced by enzyme LuxS and is associated with the periplasm by the protein LuxP. The LuxP–AI-2 complex then interacts with LuxQ. The signal three autoinducers was received by the phosphorelay protein, LuxU, and transmitted to LuxO, the DNA-binding response regulator protein. At low cell density, the autoinducers were not produced. LuxN, LuxQ and CqsA act as kinases and transfer phosphate through LuxU to LuxO. LuxO–P is active and activates transcription of genes encoding sRNAs Qrr1–5 with aid of σ^{54}. Qrr-Hfq association binds to mRNA of *luxR* and represses the translation. Thus, the LuxR protein (transcriptional regulator) is not produced and QS-induced genes are not expressed. At high cell density, the autoinducers are produced. LuxN, LuxQ and CqsA acts as phosphatases, removing phosphate from LuxU and LuxO. LuxO-P is inactive;

LuxR protein is expressed and regulates QS target genes. **(b)** Regulation of QS target genes in *V. cholerae* is similar to *Vibrio harveyi* QS. However, *Vibrio harveyi* has another VarS–VarA two-component sensory system that transmits information directly to LuxO. VarS-VarA activates transcription of some sRNAs CsrB, CsrC and CsrD that inhibit the expression of CsrA, post-transcriptional global regulator. At low cell density, VarS-VarA is absence, CsrA is active and enhances the activity of LuxO–P by some unknown component (denoted X) indirectly. HapR, the homolog of *V. harveyi* LuxR, is not expressed and not inhibit *V. cholerae* QS target genes. In the presence of VarS–VarA at high cell density, CsrA is inhibited; HapR is expressed and binds the promoters of QS target genes. The arrows indicate positive regulation, the blunt-end lines indicate negative regulation, and dotted lines indicate hypothetical interactions. OM, outer membrane; IM, inner membrane.

A homologous VarS-VarA system was found in *Pseudomonas aeruginosa*, called the 'GacS-GacA system'. GacS-GacA activates small RNAs (RsmZ and RsmY) that repress activity of translational regulator RsmA. RsmA itself regulates distinct classes of QS targets (Kay et al., 2006). This system also exists in *Erwinia* species (Barnard and Salmond, 2007).

2.16 Existence of miRNA-sized small RNAs in bacteria

The existence of non-coding RNAs with the size of miRNA (18-22 nucleotides in length) was reported in 2010 by Rao and colleagues that obtained five putative miRNAs from *Pseudomonas aeruginosa* isolated from clinical samples (Rao et al., 2010). Structural analysis of these putative miRNAs showed that they have stem-loop structure which is highly similar (~95%) to reported human miRNAs responsive to bacterial infection, using the predictive Hierarchical Hidden Markov Model (HHMM). Although these miRNAs need to be ascertained as the products from processing of longer RNAs rather than random degradation, e.g., by Northern Blot analysis, this work is considered the first to describe the preliminary characterisation of bacterial miRNAs having stem-loop structures as in eukaryotes. Later on, the term 'miRNA-size, small RNA' (msRNA), representing bacterial miRNA, was suggested by Lee and Hong that successfully identified more than 900 individual msRNA species in *Streptococus mutants* by using deep sequencing (Lee and Hong, 2012). More than 400 individual msRNA species were also proven to exist in *Escherichia coli* (Kang et al., 2013). Some of msRNAs with high

expression were verified by real time PCR and Northern Blot analysis. In addition, prediction of their secondary structures showed the characteristic of miRNA, hairpin loop with the msRNA sequence located in one strand of the duplex, using mfold software (Figure 2.16.1). These evidences confirmed the presence of miRNAs not only in eukaryotes but also in bacteria. None of the studies, however, have revealed the functional significance of the identified miRNAs that needs to be elucidated for understanding miRNA function in bacterial biology.

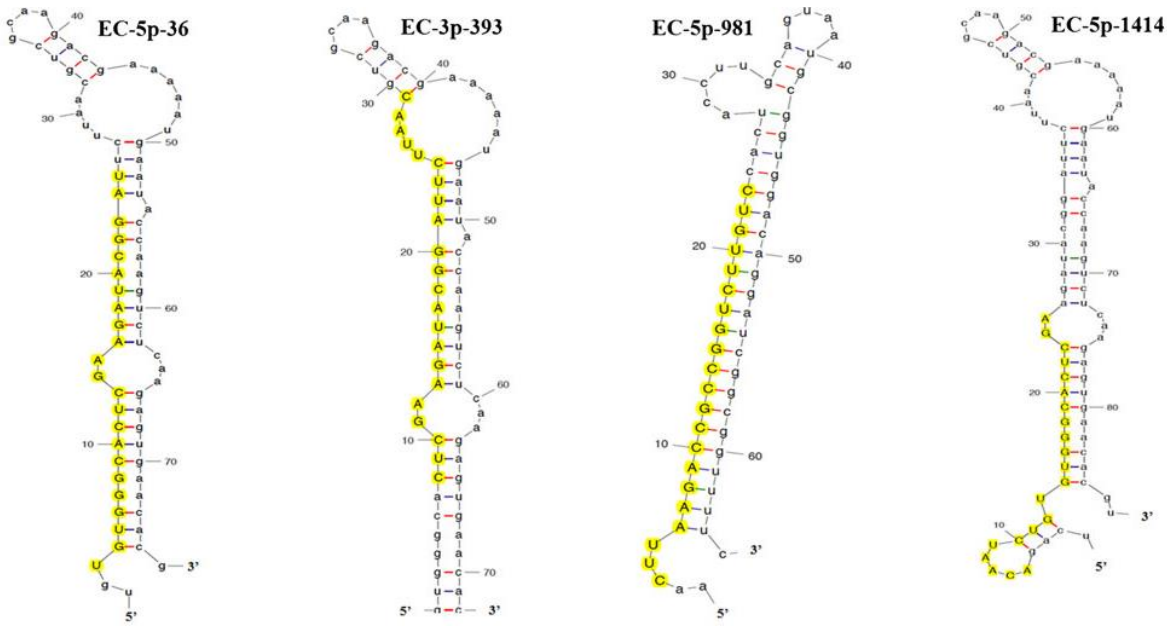

Figure 2.16.1 Stem-loop structure of some validated smRNAs in *Escherichia coli.* The smRNA sequences were highlighted in yellow (adapted from Kang et al. (2013).

2.17 Next-generation sequencing and applications

Gene expression studies are essential for interpreting the relationship between genotype and phenotype and the screening of candidate gene responses to different environmental conditions, especially at transcriptome levels, as it reveals complete set of transcripts in a cell and their abundance at specific development stage or physiological conditions (Wang et al., 2009). The reliable transcript profiling has been produced from development of novel technology such as microarray and RNA-Seq based on hybridization or sequence-based approaches, respectively.

Microarray method requires fluorescent labeled cDNA incubated with custom-made or commercial high-density oligo microarrays that have been employed to detect numerous of mRNA transcripts in diverse species such as rice (Walia et al., 2005), wheat (Kawaura et al., 2008) and barley (Walia et al., 2007) and also applied for finding miRNA population in *Arabidopsis thaliana* (Liu et al., 2008) and cotton (Yin et al., 2012). This technique has some limitations in cross-hybridization specificity, saturation of signals, sensitive gene detection in very low or high expression and requirement of up to date sequence information (Kane et al., 2000).

In contrast to the microarray method, these sequence-based approaches use the cDNA sequence directly. The method was initially reported in 1977, called 'Sanger' or 'dideoxy sequencing' that is still commonly used in DNA sequencing today (Sanger et al., 1977). Sanger sequencing usually requires amplification of the DNA fragment cloned into bacterial hosts. Thus, its application is limited to read length, requires intensive labor and cannot be applied to large sequencing projects (Hall, 2007). The development of the novel RNA-Seq method overcomes the limitations of the existing methods, allowing high-throughput sequencing. It can be used to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing) (Table 2.17.1) (Wang et al., 2009). The RNA-Seq method has advantages such as (i) its greater sensitivity, making it possible to detect genes with low expression; (ii) its ability to profile genes without prior knowledge of sequence, allowing search for novel transcripts (Zhao et al., 2014); and (iii) its improved ability to distinguish between highly similar isoforms (Wang et al., 2009). Therefore, RNA-Seq is ideal technique for gene expression analyses in organisms that lack fully sequenced genomes (Bräutigam and Gowik, 2010).

Table 2.17.1 Summary information of RNA-Seq and other methods (adapted from (Wang et al., 2009)).

Technology	**Tiling microarray**	**cDNA or EST sequencing**	**RNA-seq**
Technology specification			
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Resolution	From several to 100 bp	Single base	Single base
Throughput	High	Low	High
Reliance on genomic sequence	Yes	No	In some case
Background noise	High	Low	Low
Application			
Simultaneously map transcribed regions and gene expression	Yes	Limited for gene expression	Yes
Dynamic range to quantify gene expression level	Up to a few hundredfold	Not practical	>8,000-fold
Ability to distinguish different isoforms	Limited	YES	Yes
Ability to distinguish allelic expression	Limited	Yes	Yes
Practical issues			
Required amount of RNA	High	High	Low
Cost for mapping transcriptomes of large genomes	High	High	Relatively low

RNA-Seq can generate millions of reads, typically from 30-400 bp, depending on the platforms used (Martínez-Gómez et al., 2011). Currently, Illumina Genome Analysers (initially developed by Solexa), Roche 454 platform, Helicos and SOLiD platform from Life Technologies/Applied Biosystems (Egan et al., 2012) are used for RNA-seq since

the first introduction of next-generation sequencing in 2005 (Margulies et al., 2005). These platforms are diverse in approach, length of read and running time (Table 2.17.2) and can be used to detect novel sequences, splice variants (Howard et al., 2013) along to annotated transcripts. Among these, Illumina platform is the most widely used in genomic research. Recently, third-generation sequencing have been developed with more advance in sequencing single DNA molecule and generating long reads from 5,000 bp to 15,000 bp. Single Molecule Real Time (SMRT) (Pacific Biosciences) and MinION (Oxford Nanopore Technology) have been commercially introduced, of which SMRT is the most established platform (Lee et al., 2016).

Table 2.17.2 Information of DNA sequencing technologies.

Technology	**Approach**	**Read length**	**Bp per run**	**Time per run**
454/Roche FLX system	Pyrosequencing on solid support	200-300 bp	80-120 Mb	4 hours
Illumine/Solexa	Sequencing by synthesis with reversible terminators	30-40 bp	1 Gb	2-3 days
ABI/SOLiD	Massively parallel sequencing by ligation	35 bp	1-3 Gb	8 days
Pacific Biosciences	Sequencing by synthesis in zero-mode wave-guides (ZMWs)	10-15 kbp	1Gb	2-3 hours
Nanopore Technology	Sequencing by measuring the of electric current	5-10 kbp	10Gb	1-2 days

In general, RNA-Seq requires the RNA population to be converted to cDNA fragments, adapter ligation and attachment to one or both ends. The cDNA is then amplified, loaded onto a flow-cell and sequenced. The short sequence reads are then aligned to a reference genome of that species using programs such as Burrows-Wheeler Aligner (BWA) (Li and Durbin, 2009) or assembled into contiguous sequences (contigs) using assembly programs like ABySS (Simpson et al., 2009) and VELVET prior to aligning them to the closest possible reference sequences (Wang et al., 2009) if the complete genome is lacking. On the other hand, these short sequences can be counted for differences in expression between samples using packages such as DESeq software package (Anders and Huber, 2010). The technique has been used in diverse applications from transcriptome sequencing, such as gene expression profiling, small noncoding RNA profiling or protein coding gene annotation to epigenetic modifications of histones and DNA (Morozova and Marra, 2008). For non-coding RNA (small RNA) detection, RNA-Seq needs some modification for the preparation of samples to obtain only smaller sequences for generation of cDNA libraries (Mantione et al., 2014) (Figure 2.17.1).

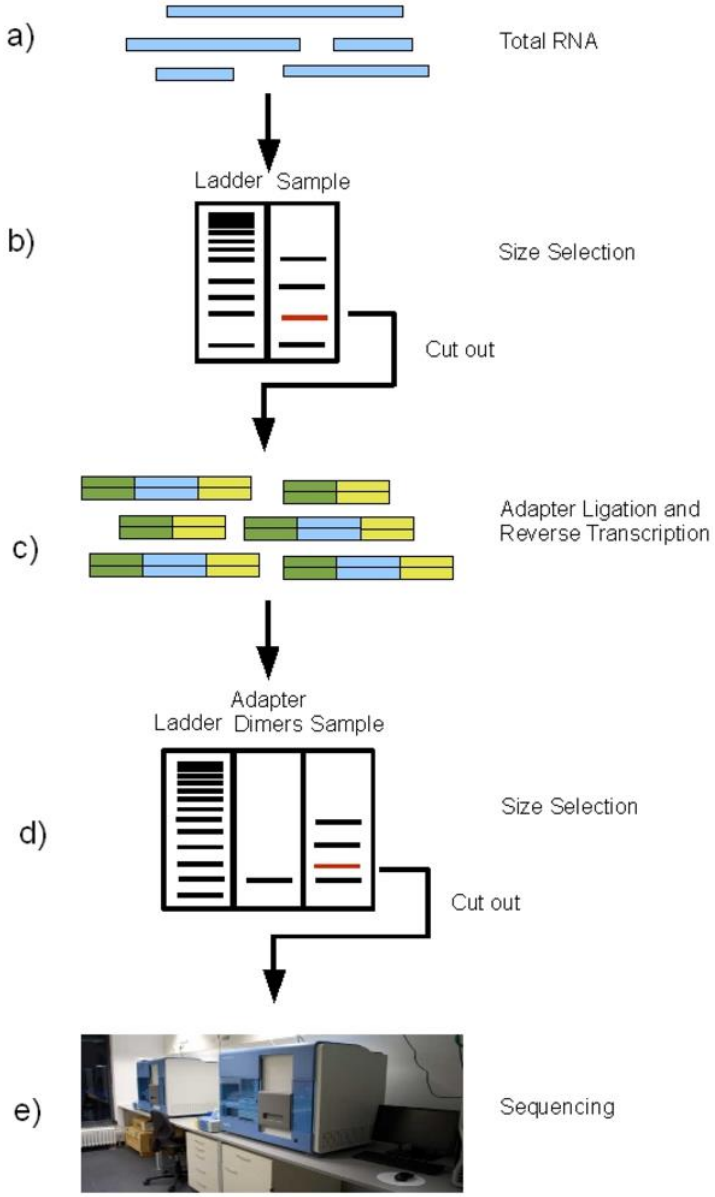

Figure 2.17.1 Summarised workflow of miRNA sequencing using Illumina Genome Analyzer (adapted from Motameny et al. (2010)).

2.18 miRNA identification and characterization

miRNAs can be identified by two different strategies: bioinformatics (computer-based) and through experimentation. Another method, forward genetics, has been employed to identify miR164c in *Arabidopsis thaliana* (Baker et al., 2005), however it is not commonly used because it is a costly and time consuming method that is limited in its ability to identify miRNAs (Budak et al., 2015).

2.18.1 Bioinformatics methods

Bioinformatics is an advanced tool for the prediction of miRNA in both animals and plants due to its benefits: low cost, high efficiency, speed and comprehensive results. The main principle of this method is to find the sequence that is homologous with the registered miRNAs in computer program. Computational analysis provides a valuable and reliable means for predicting miRNA genes of single genome or genomes over related species and their targets especially in plants where miRNA short sequences are conserved and highly complementary with their target. Therefore, mRNA sequences are also used to detect conserved miRNAs that are complementary with their targets. To date, bioinformatics is usually used to identify the sequence and secondary structure of miRNA, however this method requires the known conserved miRNA in sequence and secondary structure for identification. Therefore, this is challenge for finding new miRNAs in specific species. Many programs have been designed for miRNA identification (Unver et al., 2009) (Table 2.18.1).

Table 2.18.1 Software and their functions in miRNA identification.

Program	**Function**
miRBase (http://mivrorna.sanger.ac.uk/)	Registry for novel miRNA Providing sequence, nomenclature and references of miRNA candidates Target prediction
miRU (http://bioinfo3.noble.org/miRNA/miRU.hmt)	Finding plant target mRNA
RNA mFold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi)	Secondary structure prediction of DNA or single strand RNA.
Micro-HARVESTER (http://www-ab.informatik.unituebingen.de/brisbane/tb/index.php)	Homogenous miRNAs specificity
FindmiRNA (http://sundarlab.ucdavis.edu/mirna/)	Finding potential miRNA from given precursor sequence candidates

MiRCheck (http://web.wi.mit.edu/bartel/pub/software.html)	Checking the potential encoding miRNA of miRNA candidate

2.18.2 Experimental methods

Direct cloning is the basic method for the discovery of miRNA based on a cDNA library. This approach can be used to detect small RNAs from various tissues in different plant growth stages as well as treated plant samples under biotic or abiotic stress conditions (Unver et al., 2009).

Real-time PCR, with a stem-loop reverse transcription primer, has been developed and used for its sensitivity. miRNA was detected using a specific stem-loop primer that was hybridized to miRNA to make reverse transcription products. The reverse transcription products are then amplified by specific forward and universal reverse primers (Varkonyi-Gasic et al., 2007) (Figure 2.18.1). This method, however, is only used for detection and quantification of miRNAs, with a limitation in detecting low abundance miRNAs.

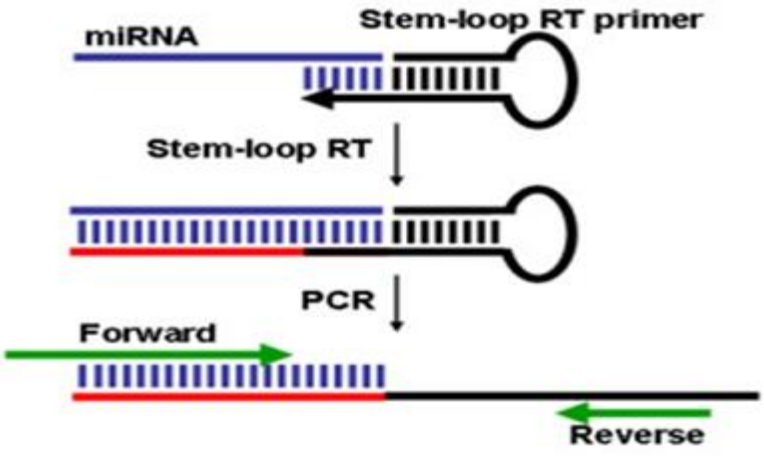

Figure 2.18.1 Principle of primer designate for miRNAs (adapted from Varkonyi-Gasic et al. (2007). 5'end of stem-loop RT primer binds to 3'end of miRNA, specific 6 nucleotides at 3' end of stem-loop RT primer pair with the last 6 nucleotides of miRNA molecule to start reverse transcription. The products continue to be amplified by the forward and universal primers.

In contrast to bioinformatics methods, experimental approaches can be used to identify novel miRNAs and miRNAs in specific species that cannot be detected using a computational approach. Hence, both experimental and bioinformatics approaches are usually applied simultaneously for the identification of miRNA species.

2.19 Prediction of small RNA targets

Identification of sRNA targets is very important for defining the function of sRNA in the life of a bacterium. Target mRNA of sRNA can be identified based on three criteria: (1) base pairing to sRNA, (2) expression of target mRNA, monitored through proteome analysis, and (3) detected by microarrays (Vogel and Wagner, 2007). Current developments in bioinformatics have been applied together with experimental methods for the prediction and validation of target mRNA samples.

Bioinformatics approaches require the availability of whole genome sequencing information for the identification of sRNA targets in bacteria. Prediction tools are usually designed for general RNA-RNA interaction or more specifically on sRNA-target RNA interactions (Li et al., 2012a). The general prediction employed by programs that find the hybridization structure of two RNA molecules do so with the minimum binding free energy such as RNAfold, Mfold, RNA cofold, RNA hybrid and RNAplex. The specific prediction models look in more detail at the flanking sequence, -200 to +200 nucleotides around the translational initiation region, seed lengths of 9 nucleotides and the loop/bulge regions from the secondary structures of sRNA such as TargetRNA, sRNATarget, IntaRNA, RNApredator or sTarpicker (Li et al., 2012a).

Experimental methods are usually used to identify the actual miRNA targets as well as validate the target mRNA from the prediction process. Some approaches have been developed for bacterial sRNA target identification, normally based on genetic and biochemical approaches. A genetic approach has been used to detect target mRNA based on the *lac* operon, wherein, if the sRNA inhibits the target genes inserted into the frame of the *lacZ* gene, the colonies will be white on X-Gal indicator plates. The target can be then identified by cloning processes. This approach was employed to find target OxyS of an sRNA upregulated under oxidative stress (Altuvia et al., 1997). Similar approaches have also been developed to detect miRNA targets in plants and animals. The transcriptome profiling is used to determine miRNA target by identifying mRNA

reduced/induced as a consequence of transfected miRNA mimics/inhibitors into the cells while translation profiling method detects the miRNA targets based on the recovery of mRNA fragment using deep sequencing when the gene transcript binds to ribosomes of cells overexpressing miRNA (Martinez-Sanchez and Murphy, 2013). Proteomic approaches identify miRNA targets by mass spectrometry of samples labelled with different isotopes (stable isotope labelling with amino acids in cell culture: SILAC) or different fluorescent dyes (two-dimensional differentiation in-gel electrophoresis: 2D-DIGE) (Thomson et al., 2011). These methods, however, cannot distinguish between direct and indirect target mRNA. A biochemical approach, in the other hand, has been developed to determine direct target mRNA. Argonaute co-immunoprecipitation was initiated to identify miRNA targets binding to epitope-tagged AGO by microarray or deep sequencing. Later on, this technique was modified with more advancement in reflection of cellular interaction and efficiency of UV crosslinking as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) and photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP), respectively (Thomson et al., 2011). In bacteria, this approach is called affinity capture, which uses sRNA as the 'bait'. Small RNAs are synthesised and labelled with biotin or a fluorescent dye. When sRNA binds to the mRNA target, the mRNA is converted to cDNA and hybridised to the whole genome. This approach has been used to identify *ompA* and *ompC* targets of RseX (Douchin et al., 2006). Another method, parallel analysis of RNA ends (PARE), is also used to identify miRNA targets by high-throughput sequencing of cleavage products from the modification of 5' RNA ligase mediated-rapid amplification of cDNA ends (5' RLM-RACE). This method is mostly applied in plant systems where the targets are subject to direct cleavage (Eckardt, 2009).

Chapter 3.

Materials and methods

3.1 Overview

This chapter describes the general and specific materials and methods adopted for investigation of miRNAs in halophilic bacteria and barley. In addition, a range of analytical techniques were used for identification and classification of marine bacteria associated to salinity as well as evaluation of barley genotypes varying salt tolerance.

Materials

3.2 Equipment

Table 3.2.1 Equipment used in this study.

Equipment	**Manufacturer**	**Purpose**
MyCycler™	Bio-Rad, California, USA	PCR, Gradient PCR
Master cycle	Eppendorf, Hambug, Germany	PCR
MyiQ™ single-color real-time PCR detection system	Bio-Rad, California, USA	Real-time PCR
Genome Analyzer IIx	Illumina	Gene expression profiling
Electrophoresis power supply-EPS 301 Minnie Gel Unit	General Electric (GE), Buckinghamshire, UK (previously Amersham Biosciences)	Separation of nucleic acids
GeneQuantTM pro UV/Vis Spectrophotometer	General Electric (GE), Buckinghamshire, UK (previously Amersham Biosciences	RNA and DNA quantification
Nanodrop	ThermoFisher, Australia	RNA and DNA quantification
MCE®-202 MultiNA, Microchip Electrophoresis System	Shimadzu, Japan	RNA quantification for mRNA-Seq
Plant growth cabinet	Thermoline, Coburg North, VIC, Australia	Growth of barley plants
Chemidoc XRS Documentation Station	Bio-Rad, California, USA	Visualisation and analysis of gel images
UV light transilluminator	UVP, USA	Visualisation of gels for gel purification
C3040 digital camera	Olympus, Tokyo, Japan	Capturing of UV exposed gel images
Finnpipette (0.5-10, 5-50, 20-200, and 100-1000 µL)	Thermo Electron, Madison, USA	Dispensing liquids

Orbital shaker/incubator	Ratek, Victoria, Australia	Bacterial growth
Varian Spectra AA220 atomic absorption spectrophotometer	Varian, Victoria, Australia	Quantitative analysis of cations

3.3 Commercial kits and solutions

Table 3.3.1 Commercial kits and solutions used in this study.

Kit/Solution	Supplier	Purpose
Wizard® Plus SV Minipreps DNA Purification System, containing cell resuspension solution, cell lysis solution, neutralization solution, column wash solution, alkaline protease solution and minicolumns	Promega, Madison, USA	Plasmid DNA purification
pGEM®-T Easy Vector System, containing pGEM®-T Easy Vector*, T4 DNA ligase, 2× rapid ligation buffer		Gene Cloning
RQ1 RNase-free DNase I	Promega, Madison, USA	Digestion of DNA contamination in RNA Samples
RQ1 DNase 10× Reaction Buffer		
Perfectprep® Gel Cleanup Kit, containing binding buffer, wash buffer, elution buffer, spin columns	Eppendorf, Hamburg, Germany	DNA purification from PCR products or from gels
TRIsure™	Bioline, London, UK	Total RNA isolation
RNase Inhibitor		Inhibition of RNase activity
Bioscript™ Moloney Murine Leukaemia Virus Reverse Transcriptase		Reverse transcription
Biomix (2×)		PCR
dNTP set		cDNA synthesis
SensiFAST™ SYBR & Fluorescein Kit		Real-time PCR
Hyperladder™ I and V		Molecular weight markers for agarose gel electrophoresis

BDT (BigDye® Terminator) v3.1 Ready Mix	Applied Biosystems, Australia	DNA sequencing
Ethidium bromide (10 mg/mL)	Sigma-Aldrich, St. Louis, USA	Staining of agarose gels
API ZYM test strips	bioMerieux, France	Enzyme test for bacterial identification
Microbact 24E Gram-negative identification system	Oxoid, UK	Bacterial identification

3.4 Preparation of solutions

3.4.1 Sterilization

Solutions were sterilized by autoclaving (121°C for 20 min), or filter sterilized through a 0.22 μM syringe filter (Millipore, Germany). All glassware and disposable plastic were also autoclaved as above. The sterilization methods used in the preparation of buffers, media and solutions listed below are indicated with autoclaved or filter sterilized.

3.4.2 Buffers and Solutions

All buffers and solutions were prepared using MilliQ water (Millipore, Germany) according to instructions as listed below.

Table 3.4.1 Composition of buffers and solutions used in this study.

Buffer/Solution	Composition	Sterilization method	Reference
TAE buffer, 50×	2.0 M Tris base, 6.5 M EDTA disodium salt, pH 8.0	autoclaved	Sambrook and Russell (2001)
Agarose gel electrophoresis loading dye, 6X	0.25% (w/v) xylene cyanol, 0.25% bromophenol blue, 30% (v/v) glycerol	autoclaved	
TB Buffer	10 mM Hepes, 15 mM CaCl2, 250 mM KCl, pH 6.7, then add MnCl2 to a final concentration of 55 mM	sterilised	
BDT reaction buffer, 5×	400 mM Tris pH 9.0, 10 mM MgCl2	autoclaved	AGRF (Australian Genome Research Facility Ltd, Melbourne, Australia)
$MgSO_4$ stock solution	0.2 mM MgSO4 in 70% ethanol	autoclaved	
Hoagland's solution	7 mM Ca(NO3)2.4H2O, 5 mM KNO3, 2 mM KH2PO4, 2 mM MgSO4.7H2O, 45 μM H3BO3, 9 μM MnCl2.4H2O, 0.7 μM ZnSO4.7H2O, 0.32 μM CuSO4.5H2O, 0.12 μM NaMoO4, 28 μM FeEDTA in 1 M KOH	autoclaved	Hoagland and Arnon (1950)
Reagent 1	45g sodium hydroxide, 150 mL methanol and 150 mL distilled water	autoclaved	Sasser (1990)
Reagent 2	325 mL certified 6.0N hydrochloric acid and 275 mL methyl alcohol	autoclaved	
Reagent 3	200 mL hexane and 200 mL methyl tert-buty ether	autoclaved	
Reagent 4	10.8g sodium hydroxide in 900 mL distilled water	autoclaved	

3.4.3 Media and solutions for microbial growth

The media and solutions used for culturing bacteria were prepared according to Sambrook and Russell (2001).

Table 3.4.2 Media and solutions for culturing bacteria.

Media/solutions	Composition	Sterilization method
Ampicilin	20 mg/mL	filter sterilization
IPTG (isopropyl-β-D-thiogalactopyranoside)	0.1 M	filter sterilization
X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)	5% (w/v) in dimethylformamide	filter sterilization
Luria broth (LB)	10 g/L tryptone, 5 g/L yeast extract, 5 g NaCl, 15 g/L agar (for plates only)	autoclaved
Super Optimal broth (SOB medium)	0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$	autoclaved
Super Optimal Broth with Catabolite repression (SOC) medium	0.5% yeast extract, 2.0% tryptone, 10 M NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose	autoclaved
Marine borth (MB)	37.4 g/L, 15 g/L agar (for plates only)	autoclaved

3.4.4 Microbial strains

Various microbial strains were used in this study, including *Escherichia coli* JM109 (Promega) and type strains of the *Thalassospira* species with validly published names (Table 3.4.3).

Table 3.4.3 Bacteria used in this study.

Bacterial strain	**Genus**	**Use**
Escherichia coli JM109	*Escherichia coli*	Molecular cloning
Thalassospira alkalitolerans JCM 18968^T	*Thalassospira*	Bacterial identification
Thalassospira mesophila JCM 18969^T	*Thalassospira*	Bacterial identification
Thalassospira lucentensis QMT2^T	*Thalassospira*	Bacterial identification
Thalassospira povalilytica Zumi 95^T	*Thalassospira*	Bacterial identification
Thalassospira profundimaris WP0211^T	*Thalassospira*	Bacterial identification
Thalassospira xiamenensis M-5^T	*Thalassospira*	Bacterial identification
Thalassospira tepidiphila 1-1B^T	*Thalassospira*	Bacterial identification
Thalassospira xianhensis P-4^T	*Thalassospira*	Bacterial identification
Thalassospira lohafexi 139Z-12^T	*Thalassospira*	Bacterial identification

3.4.5 Plant material

Seeds of *Hordeum vulgare* listed below (Table 3.4.4) were kindly provided by the Australian Winter Cereals Collection (AWCC; Tamworth, NSW, Australia).

Table 3.4.4 Barley seeds used in this study.

Cultivar/Varieties	**Species**	**Use**
Arivat	*Hordeum vulgare*	Small RNA-Seq, varietal analysis
Calmariout	*H. vulgare*	Small RNA-Seq, varietal analysis
Hindmarsh	*H. vulgare*	Varietal analysis
Mundah	*H. vulgare*	Varietal analysis
Buloke	*H. vulgare*	Varietal analysis
Vlamingh	*H. vulgare*	Varietal analysis
Skiff	*H. vulgare*	Varietal analysis
CM72	*H. vulgare*	Varietal analysis
Gairdner	*H. vulgare*	Varietal analysis
Morex	*H. vulgare*	Varietal analysis
Steptoe	*H. vulgare*	Varietal analysis
Dask	*H. vulgare*	Varietal analysis

Stirling	*H. vulgare*	Varietal analysis
Lofty Nijo	*H. vulgare*	Varietal analysis
Kaputar	*H. vulgare*	Varietal analysis

General molecular methods

3.5 Bacteria and plant propagation

3.5.1 Cultivation of bacteria for total RNA isolation

Bacteria were aerobically grown on marine borth 2216 (BD, U.S.A.) at approximately 22 - 25^{o}C for 2 days. The bacterial cultures were then centrifuged to harvest the cells for RNA extraction or stored at -80^{o}C in marine broth 2216 (BD, U.S.A.) supplemented with 20% (v/v) glycerol.

3.5.2 Growth of plants for total RNA isolation

The barley seeds were germinated on filter paper soaked with distilled water in Petri dishes at room temperature. After two days, seedlings were transferred to pots filled with a mixture of vermiculite: perlite (2:1) (Bunnings, Australia). Plants were watered with Hoagland's solution (Hoagland and Arnon, 1950) every two days and grown in plant growth cabinet (Thermoline, Australia) under conditions of 20°C temperature, 70% humidity and 12 hour day/night cycles. At two-leaf stage, three biological replicas were watered with 150 mM NaCl made in Hoagland's solution while other three used as control plants, fed with Hoagland's solution only. After 24 hours of salt treatment, leaf and root tissues were harvested separately and immediately frozen in liquid nitrogen and stored at -80^{o}C for RNA extraction.

3.5.3 Growth of plants for physiological analysis

The barley plants for physiological analysis were germinated and grown under the same conditions as above. The plants were stressed by adding 150 mM NaCl in Hoagland's solution while control plants remained unstressed in Hoagland's solution only. Leaves and roots of each plant were harvested separately at different time points, 6 h and 24 h, and used fresh for physiological analysis. Three replicates were applied for each group of each treatment.

3.6 Isolation and quantification of nucleic acids

3.6.1 Extraction of bacterial genomic DNA

For PCR amplification, DNA was isolated by using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's instruction. Briefly, 1 mL of bacterial culture was centrifuged at 12,000 rpm for 2 minutes to harvest the cells. The pellet was resuspended in 600 µL of Nucleic Lysis Solution and incubated at 80ºC for 5 minutes, then cooled to room temperature (RT). 3 µL of RNase solution was added to the suspension and the tube was incubated at 37ºC for 15-60 minutes and cooled to RT. 200 µL of Protein Precipitation Solution was added to the mixture, incubated on ice for 5 minutes and centrifuged at 12,000 rpm for 3 minutes. The supernatant was transferred to clean tube containing 600 µL of isopropanol. The tube was mixed carefully and centrifuged at 12,000 rpm for 2 minutes. The DNA pellet was washed with 70% ethanol and centrifuged at 4ºC at 12,000 rpm for 5 minutes. The pellet was air-dried and then rehydrated in 100 µL of Rehydration Solution for 1 hour at 65ºC or overnight at 4ºC.

For DNA-DNA hybridization (DDH) study, a modified CTAB method specified by the DOE joint Genome Institute (JGI, http://my.jgi.doe.gov/general/protocols.html) was used to extract the total genomic DNA. The cell pellets were resuspended in TE buffer, adjusted to OD^{600} = 1.0. 740 µL of cell suspension was transferred to a clean tube containing 20 µL lysozyme (100 mg/mL) and mixed well, After incubation at 37ºC for 30 minutes, 40 µL of 10% SDS and 8 µL of Protease K (10 mg/mL) was added to the mixture and incubated for 1-3 hours at 56ºC. Following this, 100 µL of 5 M NaCl and 100 µL of CTAB/NaCl (heated to 65ºC) were added to the suspension and incubated at 65ºC for 10 minutes. 0.5 mL of chloroform: isoamyl alcohol (24:1) was added to the above mixture, mixed well and centrifuged at max speed for 10 min at RT and the supernatant was transferred to a clean tube. This step was repeated with the addition of 0.5 mL of phenol: chloroform: isoamyl alcohol (25:24:1) and 0.5 mL of chloroform: isoamyl alcohol (24:1). The supernatant was mixed with 0.6 volume of isopropanol (-20ºC) and incubated at -20ºC for 2 hours to overnight, spinned at max speed for 15 minutes at 4ºC. The pellet was washed with cold 70% ethanol and spinned at max speed for 5 minutes. The pellet was air-dried and the resuspended in 170 µL of DNase-free water. All the purified DNA were stored at -20°C until further use.

3.6.2 Extraction of plant and bacterial total RNA

RNA was extracted using the TRIsure reagent for tissue lysis (Bioline; http://www.bioline.com/h_au.asp) according to the supplier's protocol. Fine powder of barley leaves and roots (100mg) or bacterial cells harvested from 3 mL of cultures were mixed with 1 mL of TRIsure (Bioline, Australia) and incubated at room temperature (RT) for 5 minutes. 200 µL of chloroform: isoamyl alcohol (24:1) was added to the above suspension and the tube was shaken vigorously for 15 seconds and then incubated again at RT for 3 minutes. The mixture was centrifuged at 4^{o}C at 12,000 rpm for 15 minutes. The colorless supernatant was transferred to a sterile microcentrifuge tube, mixed with 500 µL of isopropanol. The tube was kept at RT for 10 minutes and then centrifuged at 4^{o}C at 12,000 rpm for 10 minutes. The RNA pellet was washed with 75% ethanol (made with DEPC-treated water) and centrifuged at 4^{o}C at 7,500 rpm for 5 minutes. The pellet was air-dried and then dissolved in 40 µL of DEPC-treated water, incubated for 10 minutes at 60^{o}C (Sambrook and Russell, 2001). An aliquot of the RNA (5 µL) was run on agarose gel for assessing the quality and integrity of RNA preparation. The appropriate quality of RNA was then used for further analysis.

(i) Next generation small RNA-sequencing (small RNA-Seq) experiments to analyze miRNA population in marine bacteria and barley plants.

(ii) Semi-quantitative reverse transcriptase PCR (sqRT-PCR) and quantitative real-time PCR (qRT-PCR) to validate the sequencing data and to analyze different expression of selected individual genes.

3.6.3 DNase treatment of total RNA

The total RNA extracted as above was treated with DNaseI (Promega, Australia) according to the manufacturer's instructions. 35 µL of total RNA extracted was mixed with 10 units (U) of RQ1 RNase-free DNase I (Promega Australia), 5 µL of the supplied 10X reaction buffer (Promega, Australia) and 2U of RNase inhibitor (Bioline, Australia), into the final 50 µL volume with DEPC-treated water. The mixture was incubated at 37^{0}C for 30 minutes. RNA was then purified using the LiCl precipitation method according to the Ambion technical Bulletin # 160 (http://www.ambion.com/techlib/tb/tb_160.html). Briefly, 20 µL of 10 M LiCl (made in DEPC-treated water) was added to the DNase-treated RNA and the final volume made to 80 µL (with DEPC-treated water) to obtain a

final concentration of 2.5 M LiCl. The mixture was held at -20^{0}C for 30 minutes and then centrifuged at 14,500 rpm at 4^{0}C for 15 minutes. The pellet was washed twice with chilled 75% ethanol (made with DEPC-treated water), centrifuged at 14,500 rpm at 4^{0}C for 5 minutes. The RNA pellet was air-dried at RT and then resuspended into 20 µL DEPC-treated water. RNA concentrations were assessed by spectrophotometer and stored at -80^{0}C.

3.6.4 Spectrophotometric quantification of RNA

Purified RNA was quantified by recording the absorbance readings at 260 nm and 280 nm from the Nanodrop (ThermoFisher, Australia). The RNA concentrations were determined based on $1A_{260}$ = 40 µg/mL of single stranded RNA. The RNA to protein absorbance ratios (A_{260}/A_{280}) of between 1.8 and 2.0 were used as an indication of pure RNA (Sambrook and Russell, 2001).

3.6.5 Quantitation of RNA for small RNA-Seq by microchip electrophoresis

The concentration of the purified total RNA from leaf tissue of two salt-stressed and two control of two different barley cultivars was also determined for Next Generation small RNA-Sequencing (small RNA-Seq) purposes using the MCE®-202 MultiNA, Microchip Electrophoresis System (Shimadzu, Japan). The purified total RNA from two plants was diluted 1:2 with RNA marker solution. The 28S and 18S rRNA bands were recognized based on the calibration curve from the RNA 6000 ladder (Applied Biosystems). The quantitated RNA was used at the Baker IDI Heart and Diabetes Institute, Melbourne for small RNA-Seq library preparation (described in section 3.11).

3.6.6 Agarose gel electrophoresis of RNA samples

The quality of RNA (before DNase treatment) or DNA (i.e. visible bands and lack of degradation) was assessed by agarose gel electrophoresis. Agarose gels were typically prepared at 1.0-3.5% (w/v) concentration in 1X TAE buffer, with 0.5 µg/mL ethidium bromide added to the gel solution(Sambrook and Russell, 2001). Generally, 5 µL aliquots of RNA or DNA were mixed with 1 µL of 6X xylene cyanol loading dye. The DNA molecular weight markers used typically were Hyperladder™ I (200 - 1,037 bp; Bioline) or Hyperladder™ V (25 - 500 bp). Electrophoresis was accomplished using a Bio-Rad power pack at 80-100 V for 45-90 minutes. The gels were photographed on ultraviolet

transilluminator using a Chemidoc XRS Documentation Station (Bio-Rad) and Quantity One software (Bio-Rad).

3.7 cDNA synthesis

3.7.1 cDNA synthesis of mRNA

First strand complementary DNA (cDNA) was synthesized from total extracted RNA using the Bioscript MMLV reverse transcriptase (Bioline) according the supplier's instructions. 1 or 2 µg of purified total RNA was incubated with 1 µL oligo d(T)18 primer (0.5 µg/µL) (Invitrogen) at 70ºC for 5 minutes in a total volume of 12 µL making with DEPC-treated water and then chilled on ice. The mixture was added with 1 µL dNTPs (10 mM each), 10 U RNase inhibitor (Bioline), 4.0 µL of 5× reaction buffer (Bioline), 2.5 µL of DEPC-treated water and 50 U Bioscript (Bioline) and incubated at 37ºC for 1 hour. The reaction was stopped by incubation at 70ºC for 10 minutes and cDNA product was stored at -20ºC. The success and quality (lack of gDNA contamination) of the synthesized cDNA was assessed by reverse transcriptase PCR (RT-PCR) using intron-flanking actin primers and 1 µL cDNA as template.

3.7.2 Stem-loop reverse-transcription for miRNA

Stem-loop reverse transcription (RT) was carried out from total extracted RNA using stem-loop primers according to Varkonyi-Gasic et al. (2007). 1 µg of total RNA was incubated with 1 µL stem-loop primer (1 µM) (Sigma, Australia) and 0.5 µL of dNTPs (10 mM each) (Bioline, Australia) at 65ºC for 5 minutes in total of 14 µL making with DEPC-treated water and then chilled on ice for 2 minutes. The mixture was added with 4 µL of 5×reaction buffer (Bioline), 2 µL of DTT (0.1M) (Sigma), 0.1 µL of RNase inhibitor (40U/µl) (Bioline) and 0.25 µL of Bioscript (200U/µL) (Bioline). The conditions for RT reaction were followed by 30 min at 16ºC, 60 cycles of 30ºC for 30 s, 42ºC for 30 s and 50ºC for 1 s and then stored at -20ºC. The success and quality of stem-loop RT was assessed by standard PCR using specific forward primer and universal reverse primer and 1 µL of synthesized product as template.

3.8 Polymerase chain reaction (PCR)

3.8.1 Design and synthesis of primers for gene expression

Primers for polymerase chain reaction (PCR) were designed using Netprimer (http://www.premierbiosoft.com/netprimer/index.html) with the following criteria: 18-25 bases in length; maximum of 5°C difference in the annealing temperatures of the forward and reverse primers; % GC content of approximately 50% and minimal secondary structures such as hairpins, and primer dimers. The primer sequences for two housekeeping genes were designed as per Mohammadi et al. (2007) for α-tubulin and as per Hv.23088 (barley actin CDS sequence) for actin (Table 3.8.1).

Table 3.8.1 Primers used for real-time PCR in barley.

NCBI UniGene number; primer name	**Primer Sequence (5'-3')**	**Annealing temp (°C)**	**Expected cDNA amplicon size (bp)**
Target of miRNA genes			
Hv.29207 HvSPL-F HvSPL-R	 TTCTCCGATGGTCTGACTCC ATTGCTGCAGGTTGGAGAAC	54	154
KC311227 HvMyb6F HvMyb6R	 CACATGCTATACCTGGCCGA ATTGCACCATTCGTCCTCCC	54	187
Hv.9855 HvSCLF HvSCLR	 CATGGCGGAATGTGTTTGCT CCGCCATGCTGATACAGAGA	54	182
Additional genes			
Hv.31142 Hv.CMO1F Hv.CMO1R	 GTGTGTCGTCATCATGCCTC TCAGGAGGGTACCATCTAAACC	54	106
Hv.4129 HvBADH1F HvBADH1R	 GGAGCTTGGTGGCAAAAGTC CCATTGGTCCAAAAGCACCC	54	96
Hv.21574 HvABI5F	 ATCAAGAACAGGGAGTCCGC	54	189

HvABI5R	CTGCCTCTTCTTCCGTCCAA		
Housekeeping genes			
Hv.23088 HvActinF HvActinR	 TGAACCCAAAAGCCAACAGAG CACCATCACCAGAGTCGAGAAC	58	147
Hv.12354 α-tubulinF α -tubulinR	 GGACCGTACGGGCAGATCT CACCAGACTGCCCAAACACA	59	72

3.8.2 Design and synthesis of primers for miRNA amplification

Primers for miRNA amplification were designed according to the principles of Varkonyi-Gasic et al. (2007) (discussed in Chapter 2). All primers were synthesized commercially (InvitrogenTM and Sigma, Australia) and provided as dried pellets. Primers were resuspended in sterile MilliQ water to a concentration of 100 µM and stored at -20°C. The working concentration was 10 µM for all primers (Table 3.8.2).

Table 3.8.2 Primers used for miRNA amplification.

Primer name	**Primer sequences (5'-3')**
Conserved miRNA	
miR171SL miR171F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTGAG TCTCCTGTTGGCTCGACTCA
miR5048SL miR5048F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTAGAC TCGCTTATTTGCAGGTTTTAG
miR159SL miR159F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGATCAGAGC CGGCGTTTGGATTGAAGGGA
Novel miRNA	
SUT-173SL SUT-173F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCTCC TACTAGACTAGGACGCCGCC
SUT-108SL SUT-108F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCAGC TCTATCACGAGGGCTCTGCTC
SUT-174 SL SUT-174F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGAA TATTGCATCTCTCGGGTCG
SUT-45 SL SUT-45 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAATA GCGCTTCTTGCTGATGGTGT
SUT-163 SL SUT-163 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTTCT GGCCGGCATATATGTAGTGCTGTA
SUT-150 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAAAA

SUT-150 F	TGCATTTGGATCGAAGGGAG
SUT-133 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTTCAA
SUT-133 F	TGCGAACGATTTGAGGCGAT
SUT-75 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATATGC
SUT-75 F	TCAAGGAAACTGGGGCAGTG
SUT-49 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAAGA
SUT-49 F	TGTCTTCTCCGTCGACGTCA
SUT-50 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATAGA
SUT-50 F	GGCCAAACAGATCTCAAGGA
SUT-81 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGACATT
SUT-81 F	GCGCTCTTCTGAAGCTGTGG
SUT-127 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAAAA
SUT-127 F	TGCGCTTGCTCCCTTTCATT
SUT-29 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATGTA
SUT-29 F	TATACGCCGTCGCTTCGTCG
SUT-25 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCACT
SUT-25 F	TGTGTGCTTATTGACGGTCC
SUT-95 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATATGC
SUT-95 F	GCTGTTCTTCCCAGCAATGG
SUT-26 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCACT
SUT-26 F	GTCGAGCTTATTGACGGTCC
SUT-158 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTTTCT
SUT-158 F	ATACATGCATCGTGCTGGGG
SUT-84 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCTGA
SUT-84 F	TATATGCTGCGTCGACGCCA
SUT-112 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGAAA
SUT-112 F	GCTCCTACTAACGCGTTTCC
SUT-27 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGACCTA
SUT-27 F	GCGCATGGGATTGCTCGTATTA
SUT-142 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTTAAA
SUT-142 F	CGCGCGCTATGTAGACTTTTG
SUT-46 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCTCG
SUT-46 F	TGTAGGAACGTTGGCTGGCT
SUT-23 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATTTAA
SUT-23 F	CGCGCTGGGAGACTTCTAAC
SUT-94 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCCTG
SUT-94 F	TCTGTCCATCCATCCGATCC
SUT-39 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATAAAT
SUT-39 F	GCGCCGTGAATTTGTTTAACTAGA

SUT-34 SL SUT-34 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCGAGT GCGCAGCAAATGATGAGCTT
SUT-228 SL SUT-228 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCCCC GCTGCTTTGCCATCAGCCTT
SUT-79 SL SUT-79 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACAAC GCGCGAAGTTGGGCAATAAT
SUT-180 SL SUT-180 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTGGA GGCCGGCTTTCTGAACTCTTCTAT
SUT-41 SL SUT-41 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATAG ATATTGGCGGAGCTCCTGCC
SUT-135 SL SUT-135 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAATCCG GCGCATTATGAAGACCCGAT
SUT-62 SL SUT-62 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTTTA TGTCTAGCGAACGAACGATC
SUT-186 SL SUT-186 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGAGAG TATATGGCGCTCCTGCTGCG
SUT-61 SL SUT-61 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCGAC TGCTGCTAGGTTCATCCGTT
SUT-43 SL SUT-43 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTCAAC TACTAGGCGGATGTAGCCAA
SUT-10 SL SUT-10 F	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGAGA GGCCGGTTTGATTAATCCGG

3.8.3 PCR conditions

All the PCR reactions were carried out based on the principles described by Mullis and Faloona (1987). The amplifications were generally performed in 25 µL reactions containing 200 ng of gDNA, 12.5 µL of 2× Biomix (Bioline; contains Taq polymerase, $MgCl_2$, dNTPs) and 1 µL of each forward and reverse primer (10µM). Amplification for cDNA and plasmids were carried out using 20 ng cDNA or 10 ng plasmid template. Negative controls (no template) were also included. The reaction was done on the MyCycler Thermal Cycler (Bio-Rad) or Mastercycler (Eppendorf) using the cycling conditions as below (Table 3.8.3). The success of each PCR reaction was determined by running 5 µL of each PCR product on agarose gel electrophoresis.

Table 3.8.3 PCR thermal cycling conditions for mRNA genes.

Step	Temperature (ºC)	Time	Number of cycles
1. Initial denaturation	94	5 minutes	1
2. Denaturation	94	45 seconds	35
3. Annealing	Primer-specific	45 seconds	35
4. Extension	72	1 minute/kb	35
5. Final elongation	72	10 minutes	1
6. Hold	4	Hold	1

3.8.4 Semi-quantitative reverse transcriptase PCR

The technique was used for gene expression analysis. The RT-PCR reactions consisted of 1 µL of synthesized first strand cDNA as template, 12.5 µL of 2× Biomix (Bioline) and 1 µL of each primer (forward and reverse) (10 µM), in total volume of 25 µL making with sterile MilliQ water. The actin or α-tubulin primers were used as housekeeping genes. The thermal cycling conditions for PCR were identical to the typical PCR conditions (Table 3.8.3), except that each gene was amplified for 20, 25, 30, 35 cycles to determine the cycle number where the PCR is still in the exponential phase. The RT-PCR products were quantified by measuring band intensities on agarose gels. 5 µL of products were stained with ethidium bromide (0.5 µg/µL), electrophoresed on agarose gel and the intensity of bands was quantified using Chemidoc XRS Documentation Station and Quantity One software (Bio-Rad). The fold changes between control and stressed samples were calculated using the formula below (Jang et al., 2004):

$$\text{Fold change} = \frac{\text{Intensity (gene)(stress)}}{\text{Intensity (tubulin)(stress)}} \div \frac{\text{Intensity (gene)(control)}}{\text{Intensity (tubulin)(control)}}$$

3.9 Cloning and DNA sequencing

3.9.1 Purification of PCR products

PCR products were purified using Perfectprep® Gel Cleanup kit (Eppendorf, Germany) according to the supplier's protocol. Generally, 45 µL of PCR products were loaded onto agarose gel and electrophoresed. The bands were excised and mixed with 3 equivalent volumes of Binding Buffer (assuming 1 mg = 1 µL), incubated at 50ºC for 10 minutes to melt the gel. A volume of isopropanol was added, and the mixture was

transferred to a spin column assembly (provided with the kit). The spin column assembly was centrifuged at 14,500 rpm for 1 minute and then the column was washed with diluted Wash Buffer. 30 µL sterile MilliQ water was added to the column to elute the bound-DNA, incubated for 10 minutes at room temperature (RT) and then centrifuged at 14,500 rpm for 1 minute. The purified DNA was stored at -20 °C. Qualification and quantification of purified products were assessed by electrophoresis.

3.9.2 Cloning of PCR products

The PCR products were cloned into the pGEM®-T Easy Vector according to the supplier's instructions. An aliquot of purified PCR product was mixed with 1 µL of pGEM-T Easy vector, 5 µL of 2× T4 DNA Ligase Buffer and 1 µL of T4 DNA Ligase in total volume of 20 µL. Ligation mixtures were incubated at 4 °C overnight and then transformed into 100 µL competent JM109 E. coli cells.

3.9.3 Transformation of chemically competent *E. coli*

The chemically competent JM109 *E. coli* cells were prepared according to the method described by Inoue et al. (1990), with minor modifications. The JM109 *E. coli* cells from the stock culture were streaked onto a non-selective LB agar plate and grown overnight at 37°C. A single colony was picked from the plate and inoculated into 10 mL LB medium and grown overnight at 37°C with shaking (180 rpm). This culture was inoculated in 500 mL SOB media and incubated at 18°C with shaking until the cell density reached OD_{600}=0.4. The culture was kept on ice for 10 minutes and the cells were transferred to 50 mL tubes, centrifuged at 2,000 rpm for 15 minutes at 4 °C. The cells were then resuspended in 80 mL ice-cold TB buffer, incubated on ice for 10 minutes and then centrifuged as above. The cells were washed again in 20 mL TB buffer. DMSO was added to a final concentration of 7% (v/v) with gentle mixing. The cells were incubated again on ice for 10 minutes, distributed into 100 µL aliquots, immediately snap-frozen in liquid nitrogen and stored at -80 °C. The stored competent cells were thawed on ice for 5 minutes and the ligation mixture (section 2.10) was added to the cells and then held on ice for 30 min. The cells were heat shocked at 42 °C for 90 seconds and then cooled on ice for 2 minutes. 500 µL SOC was transferred to the cells and incubated at 37 °C with shaking for 1.5 hours. 100 µL of the cell suspension was subsequently plated on LB agar

containing ampicillin, IPTG and X-gal for blue-white screening. The plates were incubated at 37 °C for 18-24 hours.

3.9.4 Plasmid DNA purification

Plasmids were purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to the supplier's instructions. 'Blue-white screening' was used for selection of successful transformation. The white colony indicated recombinant clones while blue colony indicated non-recombinant clones. A single white colony was inoculated into 5 mL luria broth (LB) containing 100 µg/mL ampicillin and incubated overnight. The culture was centrifuged at 14,500 rpm for 5 minutes. The pellets were resuspended in 250 µL Cell Resuspension Solution and mixed with 250 µL Cell Lysis Solution, 10 µL alkaline protease solution. The mixture was incubated for 5 minutes at room temperature (RT). 350 µL Neutralization Solution was added and the mixture was centrifuged at 14,500 rpm for 10 minutes at RT. The supernatant was transferred to a spin column assembly (provided with the kit) and centrifuged for 1 minute at 14,500 rpm to remove all cell debris. The flow-through was discarded and the spin column was washed twice with Column Wash Solution. 50 µL of sterile MilliQ water was added to the column to elute plasmid, centrifuged at 14,500 rpm for 1 min. An aliquot of purified plasmid DNA was loaded onto agarose gel for checking quality and the concentration was determined using spectrophotometer. Purified plasmids were stored at -20 ºC.

3.9.5 DNA sequencing

The sequencing reactions were carried out using the BigDye® Terminator (BDT) v3.1 Ready Mix (Applied Biosystems) according to the instructions by Australian Genome Research Facility Ltd, Melbourne, Australia (AGRF; http://www.agrf.org.au/assets/files/PDF%20Documents/Guide%20to%20AGRF%20Sequencing%20Service.pdf). Vector-specific primer T7 (forward) or SP6 (reverse) were used for sequencing. The reaction contained 0.5 µL BDT reagent, 2 µL 5× BDT buffer, 3.2 pmol T7 or SP6 primer and 200-500 ng plasmid DNA template in total of 10 µL volume making with sterile MilliQ water. The thermal cycling conditions were as follows: an initial denaturation at 96ºC for 2 minutes, followed by 30 cycles of denaturation at 96ºC for 10 seconds, annealing at 50ºC for 5 seconds and extension at 60ºC for 4 minutes. The reaction was cleaned-up by magnesium sulphate ($MgSO_4$) according to protocol

described by AGRF. 75 µL of 0.2 mM $MgSO_4$ ethanol solution was mixed with the sample, incubated at RT for 25 minutes and centrifuged for 14,500 rpm for 15 minutes. The pellet was washed with 100 µL of 70% ethanol by centrifugation as above conditions. The pellet was air-dried and submitted to the AGRF, Melbourne for capillary separation using an AB3730xl DNA Analyzer (Applied Biosystems). The chromatograms were inspected visually for quality of the sequences using the BioEdit Sequence Alignment Editor v7.1.3 (Hall, 1999; http://www.mbio.ncsu.edu/bioedit/bioedit.html). The good chromatograms with evenly-spaced peaks, satisfactory signal intensities and minimal baseline noise were used (The University of Michigan, 'Interpretation of Sequencing Chromatograms', http://seqcore.brcf.med.umich.edu/doc/dnaseq/interpret.html; last accessed May 2014). The sequences were truncated where necessary if there is oddly-spaced intensity between peaks or sequencing reaction need to be repeated if chromatograms give ambiguous peaks.

3.10 Real time PCR

3.10.1 Design and optimization of real time PCR primers

The primers for real time PCR were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and conserved domains (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to amplify the conserved region of sequences between 75-400 bp in length, with annealing temperature of 50-65ºC. The primers were checked for minimal secondary structures such as primer dimers using NetPrimer. To optimize the annealing temperatures for each primer pair, gradient PCR was used over a 5ºC range of annealing temperature for obtaining a strong and specific band in an agarose gel. The PCR products were gel-purified and sequenced. The specificity of each primer pair was further confirmed by melting curve analysis presenting as single peaks. Melting curve analysis was also detected amplification of non-specific PCR products such as primer-dimers.

3.10.2 Real-time PCR reaction conditions

Generally, all amplifications were carried out in 20 µL reactions containing 10 µL of 2× SensiFAST SYBR & Fluorescein Mix (Bioline), 200 ng cDNA and 1 µL of each forward and reverse primer (10µM). The reactions were prepared on 96-well plates sealed with optical quality sealing tape (Bio-Rad). No template control (NTC) was also included

in 96-well plates. The reactions were analyzed on the MyiQ™ single-color real-time PCR detection system (Bio-Rad) with the cycling conditions: polymerase activation at 95ºC for 1 minute, followed by 40 cycles of denaturation (95ºC for 15 seconds), primer annealing (54ºC for 30 seconds) and extension (72 ºC for 15 seconds).

3.10.3 Data analysis

Data was collected during annealing step. Melt curve was performed by increasing the set-point temperature of 60ºC to 95 ºC by 0.5 ºC every 10 seconds. Actin and α-tubulin were used as housekeeping controls as they have relatively constant expression. Differential gene expression fold change (FC) was calculated using $2^{-\Delta\Delta C_T}$ (Livak) method (Livak and Schmittgen, 2001):

$$\Delta C_{T(test)} = C_{T(target,test)} - C_{T(ref,test)}$$

$$\Delta C_{T(calibrator)} = C_{T(target,calibrator)} - C_{T(ref,calibrator)}$$

$$\Delta\Delta C_T = \Delta C_{T(test)} - \Delta C_{T(calibrator)}$$

$$\text{Expression ratio} = 2^{-\Delta\Delta C_T}$$

3.11 Next-Generation small RNA-Sequencing

Analysis of small RNA sequences was carried out using Next-generation small RNA-Seq on an Illumina Genome Analyzer IIx (GAIIx) platform. The isolated RNAs were qualified and quantified using MultiNA bioanalyzer before preparation of the libraries. The total RNA samples were used for conducting small RNA-Seq on the Illumina GAIIx platform. The small RNA-Seq methodology consists of four main stages: (A) library preparation, (B) cluster generation, (C) sequencing and (D) data analysis. These steps were carried out with the help of the co-supervisor Dr Mark Ziemann as a part of his NGS role at Baker IDI as well as using AGRF service.

3.11.1 Library Preparation

The library preparation was conducted using NEBNext® multiplex small RNA library preparation set for Illumina (https://www.neb.com/~/media/Catalog/All-Products/FAC109E8FD1341339AEADA0A081814C7/Datacards%20or%20Manuals/m

anualE7300.pdf; accessed May 2015). Small RNA library was prepared as the following steps:

1. **3' ligation**: the qualified RNA from the above-purified DNase-treated total RNA (section 2.5.2) was ligated at 3' ends using 3'SR adaptor for Illumina and ligation enzymes.

2. **Primer hybridization**: the hybridization prevents formation of adaptor dimer using SR RT primer (Illumina) that hybridize to the excess of 3' SR adaptor, making double stranded DNA from single stranded adaptor. This step also prevents 3' SR adaptor ligation to 5' SR adaptor in the following step.

3. **5' ligation**: the RNA was ligated at 5' ends using 5' SR adaptor for Illumina and ligation enzymes.

4. **First strand cDNA synthesis**: first strand cDNA was synthesized using ProtoScript II reverse transcriptase and murine RNase inhibitor.

5. **PCR amplification**: the PCR reaction was employed to amplify cDNA template for sequencing of DNA library, containing SR primer (Illumina), Index 1 primer and LongAmp Taq 2× master mix.

6. **PCR purification and size selection**: PCR products were purified using QIAquick PCR purification kit before loading to 6% polyacrylamide gel for miRNA and piRNAs (18-35 nucleotides) selection.

3.11.2 DNA quantification and cluster generation

The libraries were quantified using MultiNA DNA500 kit. Cluster generation was performed on cBot (Illumina), a revolutionary automated system that amplified single molecule DNA template, creating clonal cluster (http://www.illumina.com/documents/products/datasheets/datasheet_cbot.pdf; accessed June 2015). This procedure allowed the immobilization (binding) of the modified fragments from the cDNA library onto the flow cell. Cluster generation consists of the following main steps: (i) Immobilization: hybridization of cDNAs to oligonucleotides immobilized on flow cell surface; (ii) 3' extension: copies of templates using hybridized primers and denaturation of the original templates to leave the copies on the flow cell

surface; (iii) Isothermal bridge amplification: hybridization of the templates loop to adjacent lawn oligonucleotides and amplification to form cluster; (iv) Linearization: removal of the reverse strand of double stranded DNA from the flow cell; (v) Blocking: blocking of the 3′ OH end of the linearized clusters; (vi) Hybridization: hybridization of the sequencing primers onto the linearized clusters.

3.11.3 Sequencing

Barcoded sequencing was performed on the Illumina Genome Analyzer IIx (GAIIx) and according to the user guide for sequencing kit v4 using a 7 cycle barcode read (published August 2009).

3.11.4 Data Analysis

Demultiplexed sequences were generated using CASAVA 1.8.2 software (Illumina). After trimming adaptor using fastx clipper and discarding sequences shorter than 18 nucleotides, image analysis, intensity scoring and base-calling was conducted using the Illumina Real-Time Analysis (v1.8) software (http://support.illumina.com/sequencing/sequencing_software/real-time_analysis_rta.ilmn).

3.12 Methods specific to Chapter 4: Description of *Thalassospira australica*

3.12.1 Phylogenetic analysis

The 16S rRNA gene sequences were amplified and sequenced by the Australian Genome Research Facility (AGRF) Laboratories (Brisbane, Australia). The 16S rRNA sequence of strain NP3b2^T was also extracted from the whole genome sequences and compared to validly described *Thalassospira* species using the CLUSTAL W program. Evolutionary phylogenetic trees were then generated using the neighbour-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum-likelihood (ML) (Felsenstein, 1981) algorithms. Genetic distances for the NJ tree were calculated using Kimura's two-parameter model (Kimura, 1980), with the robustness of 1,000 replications, using MEGA 5 software (Tamura et al., 2011).

3.12.2 Whole genome sequence analysis

The fully sequenced and assembled genome of strain NP 3b2^T was used for comparative genomic analysis (López-Pérez et al., 2014). The GC content of strain NP 3b2^T was calculated on the basis of its whole genome sequence. The complete sequence genomes of validly described *T. lucentensis* QMT2^T, *T. profundimaris* WP0211^T, *T. xiamenensis* M-5^T and *'T. permensis* SMB34^T' were retrieved from GenBank for genome analysis. Genome comparison between the strains was carried out using reciprocal BLAST analysis, according to the method described by Goris et al. (2007). The average nucleotide identity (ANI) was calculated using the JSpecies software package v1.2.1 using the default parameters (Richter and Rosselló-Móra, 2009). The in-silico genome-to-genome distance (GGD) between the five strains were also calculated using genome-to-genome distance calculator 2.0 (GGDC) provided by DSMZ, http://ggdc.dsmz.de.

3.12.3 DNA-DNA hybridization (DDH)

The high quality DNA was isolated according to the modified CTAB method (detailed in Section 3.6.1) and the concentration and purity of the samples were determined using Nanodrop (ThermoFisher, Australia). DDH was performed by quantitative real-time PCR thermocyclers according to a simple fluorimetric method (Gonzalez and Saiz-Jimenez, 2005, Rosselló-Móra et al., 2011). The samples were diluted to 0.1 µg/µL using 2X SSC buffer and 5 µL of DNA samples of each homologous and heterologous were prepared on 96-well plates in triplicate with a final volume of 10 µL. The DNA denaturation step was carried out using a MyCycler™ Thermal Cycler (Bio-Rad, USA) with the following conditions: denaturation at 99.9°C for 10 min, renaturation at the calculated T_{or} for 8 h, followed by subsequent renaturation at T_{or} - 10°C for 30 min, T_{or} - 20°C for 30 min, T_{or} - 30°C for 30 min, and hold at 15°C until further measurements.

$$T_{or} = 0.51\ (\%GC) + 47.0\ \text{(De Ley et al., 1970)}$$

10 µL of diluted SYBRR Green I nucleic acid gel stain (Invitrogen, USA) (1: 10,000) was added to each reaction mixture in the 96-well plate. Melting curves were generated using iQ™5 real-time PCR detection system (Bio-Rad, USA) with a melting ramp of 0.2°C/6 s starting at 20°C up to 99°C for 395 cycles. Melting temperature (T_m) for each sample was estimated based on the melt curves where 50% of the DNA is still

double stranded. The ΔT_m and percentage of relative binding ratio (RBR%) were calculated using the equations below.

$$\Delta T_m = T_m \text{ (homologous sample)} - T_m \text{ (heterologous)}$$

$$RBR\% = -5.0501\ \Delta Tm + 90.329 \text{ (Rosselló-Móra et al., 2011)}$$

3.12.4 Physiological and biochemical analysis

The ability of the strains to grow at various NaCl concentrations was tested using Luria-Bertani (LB) medium [per litre: 10 g tryptone, 5 g yeast extract] in the presence of 0 – 12% (w/v) NaCl and incubated at 25ºC. pH tolerance was tested at pH levels of 4.5, 5.0, 5.5, 7.0, 9.0, 10.0, 10.5, 11.0 and 12.0 by adjusting the pH of the marine agar with HCl or NaOH and incubated at 25ºC. The growth of bacteria at different temperatures was tested at 4, 10, 25, 30, 37 and 40ºC. All the tests were carried out for a period of 7 days, with results being recorded daily.

Enzymatic tests were carried out using API ZYM test strips (bioMèrieux, France). Inocula were prepared by suspending the culture that has been grown overnight into 3% (w/v) saline solution and adjusting the density of suspension to McFarland standard no. 5. Microbact 24E Gram-negative identification system (Oxoid, UK) was used to test biochemical reactions, including lysine and ornithine decarboxylase; H_2S production; glucose, mannitol and xylose fermentation; hydrolysis of o-nitrophenyl-β-D-galactopyranoside (OPNG); indole production; urea hydrolysis; acetoin production (Voges-Proskauer reaction); citrate utilization; production of indolepyruvate; gelatin liquefaction; malonate inhibition; inositol, sorbitol, rhamnose, sucrose, lactose, arabinose, adonitol, raffinose and salicin fermentation and arginine dihydrolase. All tests were performed according to manufacturer's instruction in three independent experiments and using reference type strain, *T. tepidiphila* 1-1B^T in all experiments.

3.12.5 Lipids and fatty acid analysis

Fatty acid (FA) methyl esters were prepared according to the methods described by (Sasser, 1990). 1.0 mL of Reagent 1 was added to 40 mg of bacterial cells in a clean tube. The tube was sealed, vortexed briefly and incubated in boiling water for 30 minutes with vigorous vortex for 5 – 10 seconds every 5 minutes. 2 mL of Reagent 2 was added to the above cooled mixture, vortexed and incubated at 80ºC for 10 minutes. The cooled

suspension was mixed with 1.25 mL of Reagent 3 and rotated for 10 minutes. After discarding the aqueous phase, 3 mL of Reagent 4 was added to the tube and vortexed for 5 minutes. The organic phase was pipetted into a GC vial for analysis using the standard protocol of Sherlock Microbial Identification System (version 6.0, MIDI). The resulting fatty acid methyl esters were analysed using a GC-21A chromatograph (Shimadzu) equipped with a fused-silica capillary column (30 m × 0.25 mm) coated with Supelcowax-10 and SPB-5 phases (Supelco) at 210ºC. The results were identified by using equivalent chain-length measurements and comparing the retention times to those of authentic standards.

The polar lipids were extracted according to the method described by Bligh and Dyer (1959). 1 mL of sample was mixed with 3.75 mL of chloroform: methanol (1:2). 1.25 mL of chloroform was added to the suspension and mixed well. The mixture was added with 1.25 mL of distilled water, vortexed and centrifuged at RT for 5 minutes. The bottom organic phase was used for analysis. Bacterial lipids were examined by two-dimensional thin-layer chromatography (TLC) using Kieselgel 60-HPTLC (6×6 cm) plate (Merck, Australia) in the first dimension with chloroform/methanol/water (65:25:4 by vol.) and in the second dimension with chloroform/methanol/acetic acid/water (80:12:15:4 by vol.) (Collins and Shah, 1984). The presence of lipids was visualised using spray reagents (ninhydrin, molybdite reagent, naphtol and 10% sulphuric acid in methanol).

3.13 Methods specific to Chapter 5: identification of miRNAs in bacteria

3.13.1 Bioinformatics analysis of bacterial miRNAs

The raw reads generated from the Illumina sequencing were first filtered by removing any contaminations including rDNA, Illumina smallRNA adapter sequences or low-quality reads with < 18 nt length using Cutadapt (Martin, 2011). The cleaned reads were collapsed to eliminate repeated sequences and the unique reads were then aligned against the *Thalassospira* reference genomes to map the sequences using Bowtie software with default setting (Langmead et al., 2009). The mapped sequences were used to predict miRNAs using two different miRNA prediction algorithms, miRDeep and CID-miRNA. The miRDeep software (http://www.mdc-berlin.de/rajewsky/miRDeep) was utilised to predict miRNAs based on an investigation of the secondary structure of the miRNA

precursor sequences and integration of miRNA precursors with Dicer, providing the mature miRNA sequences (Friedlander et al., 2008). CID-miRNA (http://melb.agrf.org.au:8888/) is a web-sever developed for the identification of miRNA precursors based on secondary structure filter and an algorithm of stochastic context free grammar (SCFG) (Tyagi et al., 2008). This web-sever only predicts the miRNA precursors. Therefore, *MatureBayes* (http://mirna.imbb.forth.gr/MatureBayes.html) was employed to identify mature miRNAs based on the sequence and structure of the miRNA precursors (Gkirtzou et al., 2010). These small sequences mapped to the genome were processed through miRDeep and CID-miRNA for identifying miRNAs with default parameters. The predicted miRNAs were then aligned to those reported in *Escherichia coli* DH10B and *Streptococcus mutans* ATCC 25175 (Lee and Hong, 2012, Kang et al., 2013) as well as miRNAs predicted within the genus *Thalassospira* to identify any conserved miRNAs across all bacteria within the genus.

3.14 Methods specific to Chapter 6: identification of miRNAs in barley

3.14.1 Identification of barley miRNAs

The adapter sequences were removed using Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The 18-35 nucleotide sequences were then mapped to the Bowman assembly barley genome version 5 using the Burrows-Wheeler Aligner (BWA) with default settings to eliminate any contaminated sequences. The candidates were aligned against barley miRNA sequences deposited into miRbase (miRBase v 21.0, http://www.mirbase.org/) for detecting known miRNAs. The Mireap software (http://sourceforge.net/projects/mireap/) was employed to identify novel miRNAs on the basis of the following criteria: (1) a miRNA-miRNA* duplex located in opposite stem-arms with two-nucleotide 3' overhangs, (2) mismatch of no more than 4 bases between miRNA and the other arm including miRNA*, (3) asymmetric bulges, especially within a miRNA-miRNA* duplex, of minimal size and frequency (usually less than one) (Meyers et al., 2008). The candidates were then mapped to the available genome sequences of Morex and Barke on the International Barley Sequencing Consortium (IBSC http://webblast.ipk-gatersleben.de/barley/) to detect their presence in barley species. Differential expression of miRNAs was also determined by comparing the library size adjusted read counts.

3.14.2 Computer-based identification of miRNA target genes

The putative targets of miRNAs were identified using the web-based psRNA Target Server (http://plantgrn.noble.org/psRNATarget/). The miRNA sequences were aligned to the assembled transcripts of the barley *de novo* transcriptome libraries with default parameters. Evaluation of hvu-miRNAs and their potential targets has been described by Zhang et al (Zhang, 2005). The sequences of putative targets were further analysed against *Hordeum* EST sequences at NCBI using blast searches with default setting for gene homologs for validation.

3.15 Methods specific to Chapter 7: Physiological analysis

3.15.1 Measurement of length and biomass

The barley plants were harvested as explained in section 3.5.3. The shoot and root length was measured in centimeters. Shoot and root were immediately separated and measured for fresh weight (FW). The shoots and roots were then submerged into water, incubated at 22ºC for 24 h in order to reach saturation. After measurement of turgid weight (TW), the shoots and roots were dried at 80 ºC for 48 h to record the dry weight (DW). The relative water content of the shoots was determined as per Turner (1981) as RWC= (FW-DW)/ (TW-DW).

3.15.2 Ion determination

Shoots and roots of harvested samples were rinsed under deionized water and dried at 80ºC for 2 days. Samples were added with 5 mL of 0.5 M HNO_3, incubating in a water bath at 80ºC for 2 days. Na^+ and K^+ concentration were analyzed by the atomic absorption spectrophotometer (AAS; Varian Techtron, Melbourne, Australia) (Munns et al., 2010). The standards containing 0.0 µg/ml, 0.2 µg/ml, 0.4 µg/ml, 0.6 µg/ml, 0.8 µg/ml and 1.0 µg/ml of [Na^+] or [K^+] ions. In order to suppress the ionization, KNO_3 or CsCl was added to the standards and prepared samples to give final concentration of 2000 µg/ml KNO3 for sodium suppression or 1000 µg/ml CsCl for potassium suppression. [Na^+] or [K^+] was calculated using following equation:

$$\text{mg/g DW of tissue} = \frac{\text{Concentration (mg/L)} \times \text{Sample volume (L)}}{\text{DW of tissue (g)}}$$

3.15.3 Physiology indices

The index was used to evaluate the effects of salinity on physiological parameters as calculating below (Kausar et al., 2012):

- Relative water content index: RWCI = (RWC stressed plant / RWC control plant) ×100
- Na^+ ion stress tolerance index: NaI = (Na^+ ion stressed plant / Na^+ control plant) ×100
- K^+ ion stress tolerance index: KI = (K^+ ion stressed plant / K^+ control plant) ×100
- Na^+/K^+ ion stress tolerance index: Na/KI = (Na^+/K^+ ion ratio stressed plant / Na^+/K^+ ratio control plant) ×100

Chapter 4.

Description of *Thalassospira australica* sp. nov. isolated from sea water

4.1 Declaration for Chapter 4

The results discussed in this chapter have been published as:

Ivanova EP, Lopez-Perez M, Webb HK, Ng HJ, Dang THY, Zhukova NV, Mikhailov VV, Crawford RJ, Rodriguez-Valera (2016) *Thalassospira australica* sp. nov., isolated from sea water. *Antonie van Leeuwenhoek*, 109, 1091-1100.

4.2 Overview

The genus *Thalassospira* was firstly proposed by López-López et al. (2002) for a marine bacterium isolated from the Mediterranean Sea to accommodates Gram-negative, curved to spiral rod-shaped, strictly to facultative aerobic and halophilic bacteria (Shivaji et al., 2015). To date, the genus is comprised of 9 validly named species (Euzéby, 1997) including *Thalassospira alkalitolerans*, *Thalassospira mesophila* (Tsubouchi et al., 2014), *Thalassospira lucentensis* (López-López et al., 2002), *Thalassospira povalilytica* (Nogi et al., 2014), *Thalassospira profundimaris, Thalassospira xiamenensis* (Liu et al., 2007), *Thalassospira tepidiphila* (Kodama et al., 2008), *Thalassospira xianhensis* (Zhao et al., 2010a) and *Thalassospira lohafexi* (Shivaji et al., 2015). Another two species belonging to the genus, '*Thalassospira permensis'* (Plotnikova et al., 2011) and '*Thalassospira frigidphilosprofundus'* (Pulicherla et al., 2013) were isolated from soil and Bengal Bay water, respectively; however, their taxonomic status remains to be validated.

Members of the genus *Thalassospira* were reported to be able to utilise a range of hydrocarbons, especially plastic, as their sole carbon and energy sources (Gauthier et al., 1992, Zhou et al., 2016, Kodama et al., 2008, Nogi et al., 2014). The ability to degrade polycyclic aromatic hydrocarbons and polyvinyl alcohol was reported for *T. tepidiphila* 1-1B^T and *T. povalilytica Zumi* 95^T, respectively (Kodama et al., 2008, Nogi et al., 2014). Members of the genus can tolerate up to 10% (w/v) NaCl (Shivaji et al., 2015) and are considered a small group in comparison to other bacterial communities. In this chapter, two marine bacteria, NP 3b2^T and H 94, isolated during the course of a taxonomic survey of marine bacteria, were classified as novel species of the genus *Thalassospira* based on a polyphasic taxonomic approach.

4.3 16S rRNA gene sequence analysis

The complete 16S rRNA gene sequences of strains NP 3b2^T and H 94 were initially determined as described in Chapter 3. A phylogenetic analysis of the 16S rRNA genes was carried out using the neighbour-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum-likelihood (ML) (Felsenstein, 1981) algorithms. All three algorithms were generated using MEGA 5 software (Tamura et al., 2011) in which genetic distances for the NJ and ML trees were calculated using Kimura's two-parameter model (Kimura, 1980).

Based on the neighbour-joining phylogenetic tree, strains NP 3b2^T and H 94 were grouped in a coherent cluster (bootstrap value of 96%), forming a separate branch with *T. povalilytica* Zumi 95^T, *T. profundimaris* WP0211^T and *T. tepidiphila* 1-1B^T (with a bootstrap value of 84%) (Figure 4.3.1). The phylogenetic trees based on the 16S rRNA gene sequences, which were constructed using maximum-parsimony and maximum-likelihood algorithms, both showed similar topologies to the neighbour-joining tree (Figure 4.3.1). The observation allowed the proposal to be put forward that the newly isolated strains NP 3b2^T and H 94 belonged to genus *Thalassospira.*

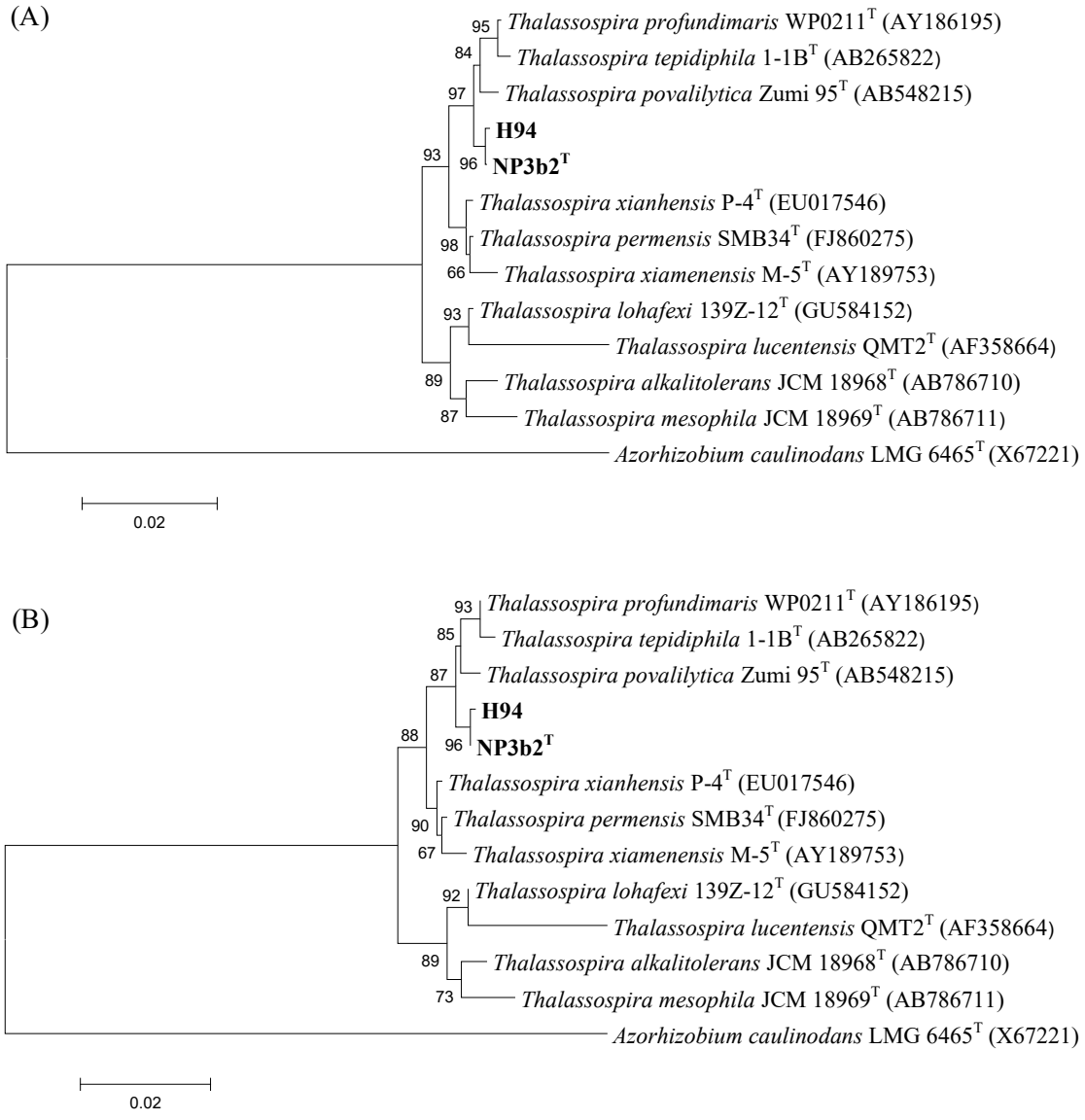

Figure 4.3.1 Taxonomic position of strains NP3b2^T, H94 and other species of the genus *Thalassospira* based on the 16S rRNA gene sequence similarities from **(A)** the neighbour-joining (NJ) phylogenetic tree and **(B)** the maximum-likelihood (ML) algorithms. The *Azorhizobium caulinodans* LMG 6465^T (X67221) was used as the outgroup in the analysis. The numbers given at the branching points are percentage bootstrap values based on 1000 replications, with only values above 50% being shown. The scale bar represents 0.02 substitutions per nucleotide position.

In order to support the proposal of NP 3b2^T and H 94 being novel species of the genus *Thalassospira*, a sequence similarity analysis of the 16S rRNA genes between NP 3b2^T, H 94 and other described *Thalassospira* species was carried out. The sequence similarity of the 16S rRNA gene between NP 3b2^T and H 94 was found to be 99.87%. Both strains shared 98-99% 16S rRNA similarity with three phylogenetically closest species, *T. povalilytica* Zumi 95^T, *T. profundimaris* WP0211^T and *T. tepidiphila* 1-1B^T, and 95-98% with the rest of the validly published species (Table 4.3.1).

Species differentiation based on sequence similarity of the 16S rRNA gene has long been discussed for limitations with regard to conservation among species, nucleotide variation in multiple rDNA regions and the possibility of 16S rRNA being produced from a horizontal gene transfer process (Zhi et al., 2012). The threshold value was originally suggested to be 97% (Stackebrandt and Goebel, 1994), but was recently changed to 98.7-99% (Stackebrandt and Ebers, 2006). High 16S rRNA gene sequence similarity, however, still occurred between the two distinct species in the genus *Thalassospira*, e.g., *T. profundimaris* WP0211^T and *T. povalilytica* Zumi 95^T (99.45%) or *T. xianhensis* P-4^T and *T. xiamenensis* M-5^T (99.30%). Hence, the genomic analysis together with physiological and biochemical properties will be further discussed in this chapter for the determination of taxonomic positions of strains NP 3b2^T and H 94.

Table 4.3.1 16S rRNA sequence similarities for strains NP3b2^T and H94 and the type species of the genus *Thalassospira*.

Species name	**Similarity of 16S rDNA (%)**											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
1.Strain NP 3b2^T	100											
2. Strain H 94	99.87	100										
3. *T. alkalitolerans* JCM 18968^T	98.02	97.88	100									
4. *T. lucentensis* QMT2^T	95.09	95.09	95.84	100								
5. T. *mesophila* JCM 18969^T	97.53	97.39	98.73	95.33	100							
6. T. *povalilytica* Zumi 95^T	99.45	99.31	97.95	94.88	97.67	100						
7. T. *profundimaris* WP0211^T	99.38	99.24	97.88	94.88	97.32	99.45	100					
8. T. *tepidiphila* 1-1B^T	98.73	99.01	97.63	94.78	97.05	99.08	99.29	100				
9. T. xiamenensis M-5^T	98.68	98.54	97.74	94.73	97.39	98.53	98.47	97.80	100			
10. T. xianhensis P-4^T	99.04	98.90	98.02	94.95	97.81	98.90	98.90	98.16	99.30	100		
11. 'T. permensis' SMB34^T	99.07	98.93	98.00	95.00	97.78	98.93	98.86	98.62	99.50	99.79	100	
12. 'T. lohafexi' 139Z-12^T	98.46	98.54	99.09	96.41	98.52	98.14	98.08	97.45	97.91	98.28	98.21	100

4.4 Phenotypic analysis

A phenotypic analysis is one of the oldest tools used in bacterial taxonomy. The analysis is based on the examination of the morphological, physiological and biochemical properties of bacterial strains (Vandamme et al., 1996). In this study, phenotypic analyses were performed for strains NP 3b2^T and H 94. *T. tepidiphila* 1-1B^T was included in the study, as it was the phylogenetically closest species that was suggested to use as reference strain by Tindall et al. (2010).

4.4.1 Morphology

The morphological characteristics of strains NP 3b2^T and H 94 were examined after 2 days of incubation in Marine Broth 2216 at 25ºC using light and scanning electron microscopy. The newly isolated bacteria have a cell size of 0.3 × 1.6 µm for the NP 3b2^T and 0.3 × 1.5 µm for the H 94 strain, respectively **(**Figure 4.4.1**).** These strains were found to be Gram-negative, aerobic, curved to spiral organisms, forming semi-translucent, non-pigmented, circular and smooth colonies after 2 days of incubation on Marine Agar 2216 at 25ºC.

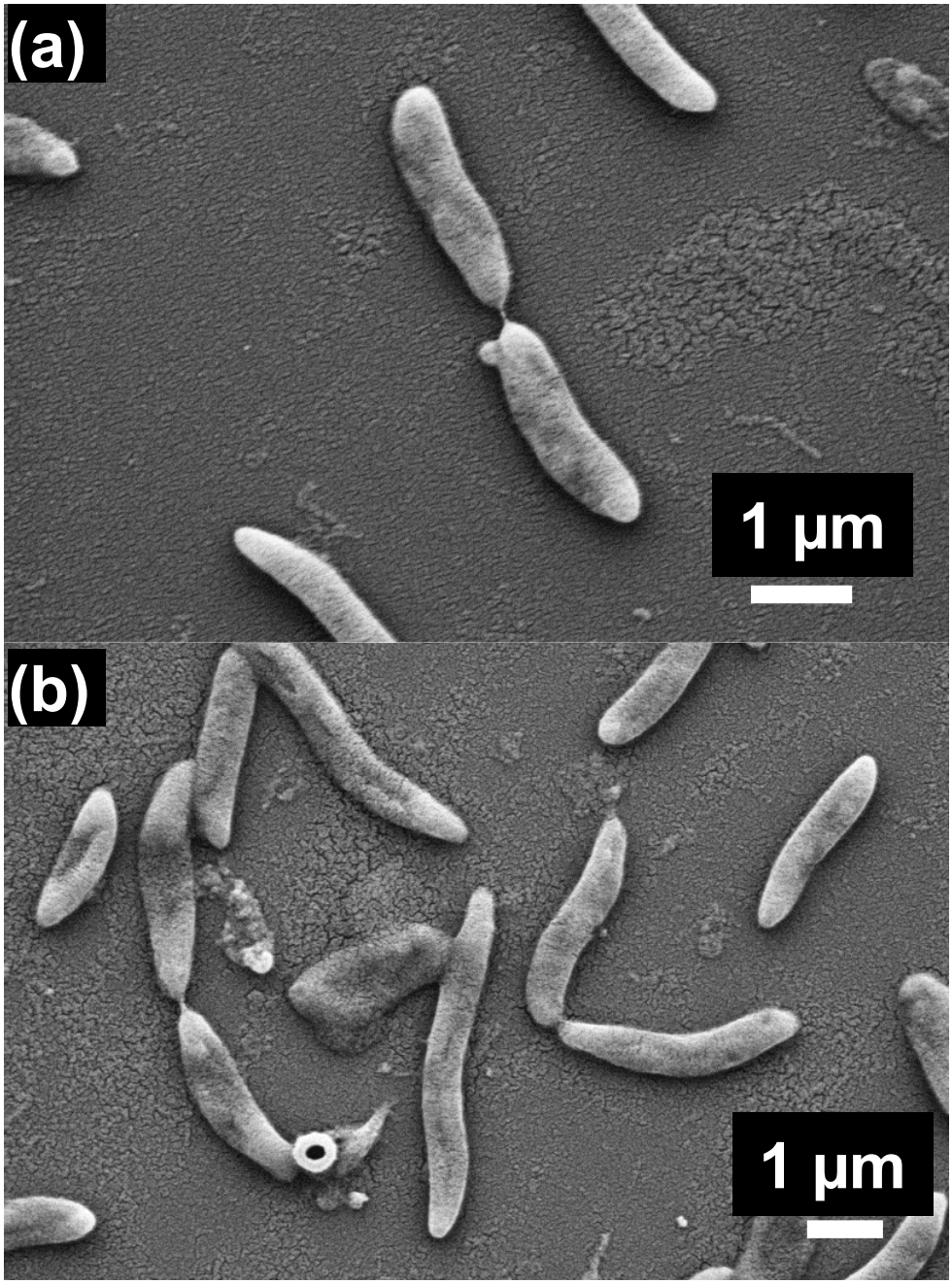

Figure 4.4.1 Scanning electron micrographs of cells of strains (a) H 94 and (b) NP 3b2^T.

4.4.2 Biochemical, physiological and metabolic characteristics

Strains NP 3b2^T and H 94 were examined for their growth under a range of temperature, salinity and pH, with the results being recorded daily over a 7 day period. Both strains exhibited their growth to a temperature range between 4 and 40ºC. The growing range of salinity for these strains was observed between 1 and 10% (w/v) NaCl while the pH range was found to be in 5 to 10. These characteristics are similar to those of the

Thalassospira spp., which can tolerate temperatures up to 40ºC, salinity up to 10% (w/v) NaCl and pH levels up to 10 (Table 4.4.1).

In order to test the physiological and biochemical properties of strains NP 3b2^T and H 94, a Microbact TM 24E Gram-negative identification system (Oxoid, UK) and API ZYM test strips (bioMerieux, France) were used. The tests were performed in duplicate for NP 3b2^T, H 94 and the phylogenetically closest species *T. tepidiphila* 1-1B^T. Both bacteria were found to produce alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase but did not produce lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β- galactosidase, β- glucuronidase, α- glucosidase, β- glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase or α-fucosidase, and were negative for H_2S and indole production (Table 4.4.1).

As seen from the data presented in Table 4.4.1, strains NP 3b2^T and H 94 exhibited similarity in most of their phenotypic characteristics. Both strains, however, showed major differences in the phenotypic features of 9 validly described *Thalassospira* species. The results suggested that both strains NP 3b2^T and H 94 belonged to the same species of the genus *Thalassospira*, which is in agreement with the results from the analysis of phylogenetic trees. Notably, some results obtained from the reference strain, *T. tepidiphila* 1-1B^T, were not consistent with the previously published data. This discrepancy was also observed in the *Marinobacter sp* study performed by Ng et al. (2014), who suggested that the differences may have been due to differences in the cultivation and incubation conditions being used.

Table 4.4.1 Differential phenotypic characteristics in strains NP 3b2^T and H 94 and other type strains of the *Thalassospira* genus.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
NaCl concentration (%)											
Range	2 - 10	1 -10	0 - 10	2 - 10	1 - 11	1 - 12	1 - 10	1 - 12	0.5 - 10	0.5 - 12	0.5-11
Optimum	4	4	2 - 4	4	2	4	2 - 3	2 - 3	2 - 3	3 – 4	ND
pH	5.5 - 10	5 - 10	5 - 10	5 - 10	4.5 - 11	4.0 - 10.5	ND	5.0 - 10.0	ND	5.5 - 9.0	ND
Temperature (°C)											
Range	4-40	4-40	8-46	4-40	4-38	4-36	10-37	4-40	4-40	10-42	4-37
Optimum	37	37	43	ND	ND	ND	22	25	22	30	25
Gelatinase	-	-	-	-	-	-	-	-(-)	-	-	+
Nitrate reduction	+	+	+	-	-	-	+	+(+)	+	+	-
Nitrite reduction	-	-	ND	ND	ND	ND	ND	-	ND	ND	ND
Utilization of											
Citrate	-	-	ND	+	W+	+	-	+(+)	-	+	+
L-Arabinose	-	-	+	-	-	-	ND	+(-)	+	+	+
Glucose	-	-	+	+	-	-	+	+	+	+	-
Glycerol	-	-	+	+	+		+	w+(-)	+	+	-
Inositol	-	-	ND	-	-	W+	-	+	+	+	-
Lactose	-	-	+	+	-	+	-	+	-	+	-
Maltose	-	-	-	+	-	+	-	-	+	+	-
D-Mannitol	-	-	+	-	-	-	+	-	+	+	-

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Sucrose	-	-	-	-	-	+	-	-	+	+	-
Ornithine	-	-	-	+	-	+	-	-	+	+	-
N-acetyl-β-glucosaminidase	-	-	ND	+	-	-	ND	-(-)	+	ND	ND

Strains: 1, strain NP3b2^T (data from this study); 2, H94 (data from this study); 3, *T. povalilytica* JCM 18746^T; 4, *T. lucentensis* DSM 14000^T; 5, *T. alkalitolerans* JCM 18968^T; 6, *T. mesophila* JCM 18969^T; 7, *T. profundimaris* DSM 17430^T; 8, *T. tepidiphila* JCM 14578^T (data from this study and Kodama et al. 2008); 9, *T. xiamenensis* DSM 17429^T; 10, *T. xianhensis* JCM 14850^T; 11, *T. lohafexi* 139Z-12^T.

The data in brackets are from this study

+, Positive; -, Negative; w, Weak reaction; ND, no data available

All species are Gram-negative, positive for citrate and negative for indole, gelatin, arginine and H_2S production

4.4.3 Chemotaxonomic analysis

Fatty acid and lipid content represent other taxonomic criteria for the identification and classification of bacteria (Vasyurenko and Frolov, 1986). Analysis of the fatty acid content performed using the Sherlock Microbial Identification System (version 6.0, MIDI) (as described in Chapter 3) revealed that the major fatty acids in NP 3b2^T and H 94 were C18:1ω7c, C16:0, C16:1ω7 and C17:0 cyclo, the main cellular fatty acids typical to the genus *Thalassospira* (Table 4.4.2).

Polar lipids of two isolated bacteria were examined by two-dimensional thin-layer chromatography (TLC) (as described in Chapter 3). Phosphatidylethanolamine, phosphatidylglycerol, glycolipid, unknown aminolipid and unknown lipid were identified as being the main polar lipids present, which is in consistent with the findings from other validly described species of the genus *Thalassospira* (Figure 4.4.2).

Table 4.4.2 Fatty acid content of strains NP 3b2^T and H 94 and other validly named *Thalassospira* species.

Fatty acid	NP3b2^T	H 94	*T. lucentensis* DSM 1400^T	*T. povalilytica* JCM 18746^T	*T. profundimaris* WP0 211^T	*T. tepidiphila* JCM 14578^T	*T. xiamenensis* M-5^T
iso-$C_{15:0}$	0.48	0.48	0.42	0.61	0.40	0.57	0.37
cyclo-$C_{17:0}$	**4.40**	**4.06**	**5.13 (4.77)**	**3.19 (1.30)**	**10.42 (5.69)**	**4.59 (9.30)**	**3.68 (1.37)**
cyclo-$C_{19:0}$	0.71	1.29	0.63	3.82	6.87	7.44	0.52
$C_{12:0}$	0.22	0.18	0.29	2.43	0.30	0.47	0.72
$C_{14:0}$	3.59	3.60	3.38	4.33	3.65	5.03	6.76
$C_{15:0}$	0.10	0.18	0.13	ND	0.13	0.20	0.31
$C_{16:0}$	**12.50**	**14.33**	**13.48 (15.77)**	**10.92 (14.20)**	**17.35 (17.79)**	**13.97 (20.25)**	**13.42 (14.89)**
$C_{17:0}$	0.14	0.18	0.20	ND	0.12	ND	0.13
$C_{18:0}$	1.47	2.73	1.98	3.54	0.80	3.05	1.32
$C_{15:1\omega6}$	0.76	0.75	0.70	0.93	0.57	1.05	0.57
$C_{16:1\omega7}$	**12.07**	**11.16**	**8.91 (15.95)**	**5.94 (5.30)**	**10.54 (2.65)**	**6.61 (4.67)**	**11.38 (7.89)**
$C_{16:1\omega5}$	0.55	0.41	0.50	0.50	0.16	0.35	0.56
$C_{18:1\omega9}$	0.52	0.44	0.16	0.48	0.54	0.36	0.47
$C_{18:1\omega7}$	**49.14**	**45.55**	**50.74 (45.25)**	**46.91 (64.60)**	**33.68 (43.13)**	**38.28 (41.73)**	**45.81 (44.34)**

Fatty acid	**NP3b2^T**	**H 94**	***T. lucentensis* DSM 1400^T**	***T. povalilytica* JCM 18746^T**	***T. profundimaris* WP0 211^T**	***T. tepidiphila* JCM 14578^T**	***T. xiamenensis* M-5^T**
$C_{18:1\omega5}$	0.38	0.24	ND	0.54	0.34	0.33	0.37
$C_{20:1\omega7}$	0.20	0.18	0.21	0.54	0.12	ND	ND
$C_{14:0}$-3OH	3.64	4.47	3.88	3.47	5.48	5.75	5.39
$C_{16:0}$-3OH	7.37	8.83	7.59	8.31	7.06	9.62	7.45
$C_{18:0}$-3OH	1.16	0.89	0.95	3.45	0.57	2.21	0.75

ND, not detected

The data in brackets are from previous studies

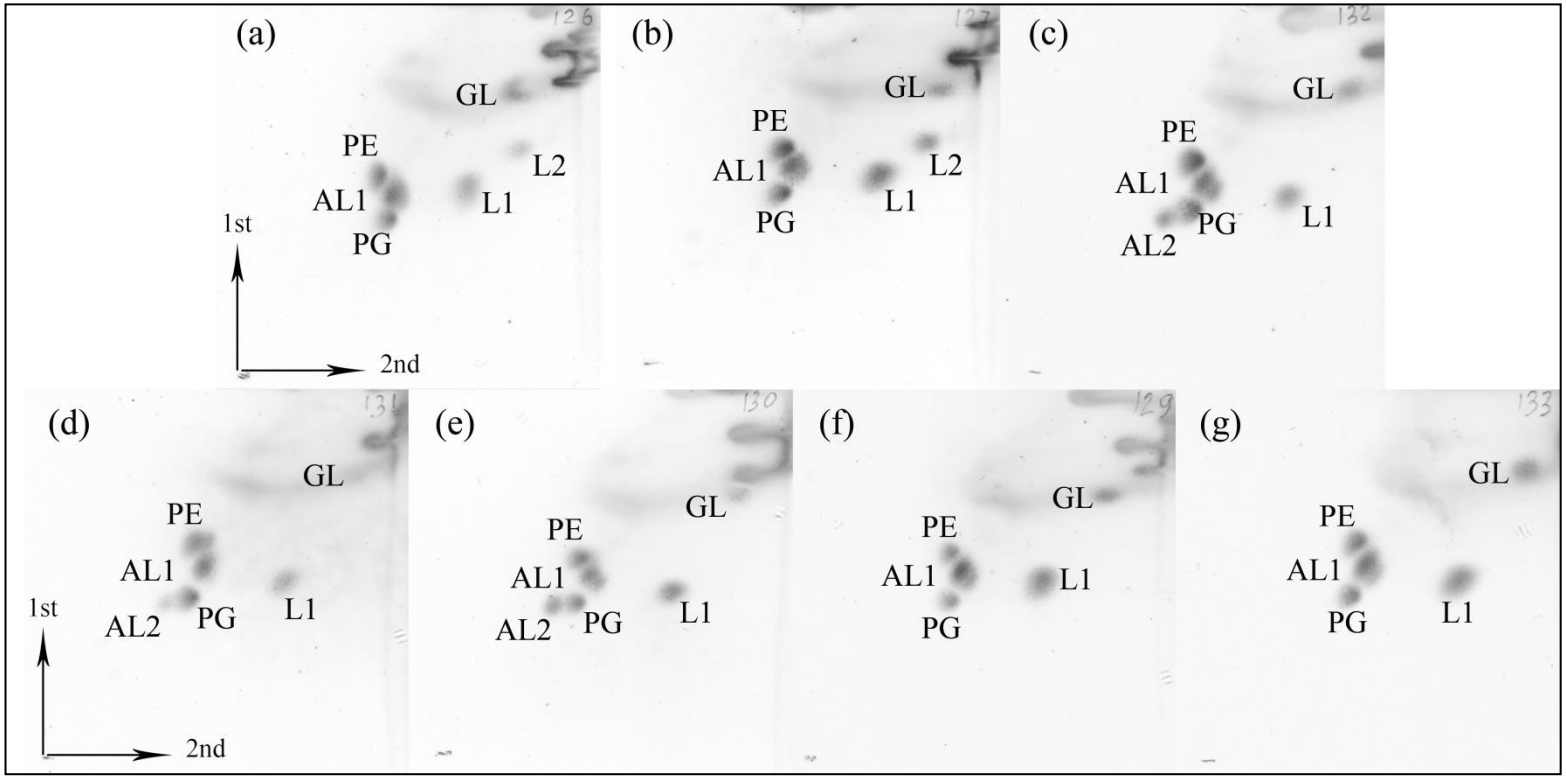

Figure 4.4.2 Two-dimensional thin layer chromatography of lipid analysis from strains (a) NP3b2^{T} and (b) H94 and other validly named *Thalassospira* species, (c) *T. tepediphila*, (d) *T. profundimaris*, (e) *T. povalilytica*, (f) *T. lucentensis*, and (g) *T. xiamenensis*. 1st: the first dimension with chloroform/methanol/water (65:25:4 by vol.), 2nd: the second dimension with chloroform/methanol/acetic acid/water (80:12:15:4 by vol.). PE, phosphatidylethanolamine; PG, phosphatidylglycerol; GL, glycolipid; AL1 and AL2, unknown aminolipids; L1 and L2, unknown lipids.

4.5 Genotypic analysis

Apart from phylogenetic and phenotypic analyses, the genotypic characteristics of a strain are considered to be crucial criteria in studies of bacterial taxonomy, including G+C content and DNA-DNA hybridization (DDH). DDH is genomic gold standard for bacterial species definition, with a DDH value lower than 70% resulting in the species being considered as distinct (Wayne et al., 1987) whereas to be put forward that the DNA G+C content is a taxonomic marker that remains fairly constant within a group. In preference to DNA sequencing, whole genome sequence analyses have been suggested as being appropriate for bacterial taxonomy (Kim et al., 2014) as this can provide the G+C content and Average Nucleotide Identity (ANI) that may suitably substitute for the DDH analysis (Tindall et al., 2010). In this study, the whole genome sequence of the type strain was also analysed for integration into the bacterial taxonomic system.

4.5.1 DNA-DNA hybridization

In order to confirm the taxonomic standing of the newly isolated strains as being novel species, a DNA-DNA hybridization was carried out between these strains, and the type strains of other species of the genus *Thalassospira*. A fluoroimetric method was employed for DDH analysis using a quantitative real-time PCR thermocycler (Loveland-Curtze et al., 2011, Gonzalez and Saiz-Jimenez, 2005). A high purity genomic DNA extract was obtained using the modified CTAB method (as described in Chapter 3) with the A_{260}/A_{280} ratio between 1.8 to 2.0 being used to assess the DDH performance in triplicate. The genomic relatedness between strains NP 3b2^T and H 94 was found to be 80.2%, whereas it was found to be only 53-65% with other type strains of the genus (Table 4.5.1). The results confirmed that NP 3b2^T and H 94 represent the same geno-species, which is novel to other valid species of the genus *Thalassospira* based on a DDH threshold value being lower than 70% being applied for species differentiation (Wayne et al., 1987). As can be seen from the data presented in Table 4.5.1, the DDH relatedness values obtained in this study were quite different to that of previously reported data, possibly being due to the difference in method used for assessment of DDH performance.

Table 4.5.1 Genetic relatedness of strain NP 3b2^T and other validly named *Thalassospira* species.

	1	2	3	4	5	6	7	8	9	10	11
1. NP 3b2 ^{T}	100										
2. *T. alkalitolerans* JCM 18968^T	55.5	100									
3. *T. lucentensis* QMT2^T	61.0	(12.5 - 16.0)[3]	100								
4. *T. mesophila* JCM 18969^T	58.5	(7.3 - 15.1)[3]	(7.1 - 11.0)[3]	100							
5. *T. povalilytica* Zumi 95^T	60.5	ND	ND	ND	100						
6. *T. profundimaris* WP0211^T	60.5	ND	(26)[7]	ND	(30.3 - 36.1)[2]	100					
7. *T. tepidiphila* 1-1B^T	53.5	(11.3 - 19.4)[3]	(17.2 - 24.2)[3]; (32.0)[4]	(9.0 - 10.4)[3]	(23.4 - 33.4)[2]	(50.7)[4]; (49.3)[6]; (69.4 - 79.6)[2]	100				
8. *T. xiamenensis* M-5^T	59.0	(24.1 - 25.0)[3]	(15.2 - 19.4)[3]; (21 – 23)[7]	(8.0 - 15.8)[3]	(14.0 - 21.8)[2]	(13.9 - 24.7)[2]; (62 – 63)[7]; (37.4)[6]	(12.6 - 23.9)[3]; (15.4 - 16.2)[2]; (35.0)[6]; (35.7)[4]	100			
9. *T. xianhensis* P-4^T	58.5		(5.3)[5]		(16.5 - 17.9)[2]	(8.3)[5]; (14.7 - 23.5)[2]	(28.3)[5]; (13.1 - 17.5)[2]	(36.0)[5]; (43.4 - 49.8)[2]	100		
10. '*T. permensis*' SMB34^T	ND	ND	ND	ND	ND	(34.5)[6]	(34.7)[6]	(46.8)[6]	ND	100	

	1	2	3	4	5	6	7	8	9	10	11
11. '*T. lohafexi*' 139Z-12^T	65.0	(31.5)[1]	(44.5)[1]	(22.0)[1]	(33.0)[1]	(12.0)[1]	(58.5)[1]	(9.5)[1]	(37.0)[1]	(15.0)[1]	100

ND, data not available;

The data in brackets are from previous works; 1 Shivaji et al. 2015; 2 Nogi et al. 2014; 3 Tsubouchi et al. 2014; 4 Kodama et al. 2008; 5 Zhao et al. 2010; 6 Plotnikova et al. 2011; 7 Liu et al. 2007.

4.5.2 Whole genome sequence analysis

Based on the DDH analysis, strains NP 3b2^T and H 94 can be classified as belonging to the novel species of the genus *Thalassospira*. Hence, the whole genome sequence was only determined for NP 3b2^T as the type strain. Analysis of the whole genome of strain NP 3b2^T revealed a genome 4,268,334 bp in size, comprised of 32 contigs and with a G+C content of 53,6 %. (López-Pérez et al., 2014). The NP 3b2^T strain possessed 3,934 predicted genes, 3875 putative coding sequences (CDS), 5 rRNAs and 55 tRNAs (Table 4.5.2). Compared to availability of whole genome sequences for the other four *Thalassospira* species, the size and GC content of these strains are very similar, confirming that the NP 3b2^T strain belonged to the genus *Thalassospira* due to a fairly constant GC content in the group, as suggested by Tindall et al. (2010). A notable difference, however, was found in number of genes assigned to the category "Nitrogen metabolism", *i.e.*, '*T. permensis*' SMB34^T and *T. xiamenensis* M-5^T possessed genes for denitrification (reduction of nitrates back into the largely inert nitrogen gas) (Table 4.5.2).

Table 4.5.2 Genomic characteristics of strain NP 3b2^T and other available genome *Thalassospira* species.

Characteristics	**NP 3b2^T**	***T. lucentensis* QMT2^T**	**'*T. permensis*' SMB34^T**	***T. profundimaris* WP0211^T**	***T. xiamenensis* M-5^T**
GenBank accession numbers	JRJE00000000.1	ATWN00000000.1	AUNC00000000.1	AMRN00000000.1	CP004388
Sequence size (bp)	4,270,334	4,748,691	4,433,312	4,380,232	4,705,237
Number of contigs	32	23	72	28	76
GC content (%)	53.6	53.4	54.8	55.2	54.6
Shortest contig size	1039	2437	243	392	500
Median sequence size	46846	138734	28536	54278	20237
Longest contig size	788206	879648	502862	910770	709434
Number of Coding Sequences	4,046	4,515	4,271	4,124	4,475
SEED classification					
Cofactors, Vitamins, Prosthetic Groups, Pigments	268	286	286	279	285
Cell Wall and Capsule	101	125	108	104	111
Virulence, Disease and Defence	61	82	73	85	75

Characteristics	**NP 3b2^T**	***T. lucentensis* QMT2^T**	**'*T. permensis*' SMB34^T**	***T. profundimaris* WP0211^T**	***T. xiamenensis* M-5^T**
Potassium metabolism	16	21	25	14	25
Photosynthesis	0	0	0	0	0
Miscellaneous	30	32	33	28	33
Membrane Transport	200	240	216	201	216
Iron acquisition and metabolism	31	28	31	42	37
RNA Metabolism	167	127	173	123	175
Nucleosides and Nucleotides	103	116	115	113	115
Protein Metabolism	243	245	227	240	209
Cell Division and Cell Cycle	26	26	26	26	29
Motility and Chemotaxis	110	100	99	85	110
Regulation and Cell signalling	84	96	86	83	86
Secondary Metabolism	5	8	5	5	5
DNA Metabolism	75	73	74	71	87
Fatty Acids, Lipids, and Isoprenoids	149	158	147	133	145

Characteristics	**NP 3b2^{T}**	***T. lucentensis* QMT2^{T}**	**'*T. permensis*' SMB34^{T}**	***T. profundimaris* WP0211^{T}**	***T. xiamenensis* M-5^{T}**
Nitrogen Metabolism	17	22	70	27	70
Dormancy and Sporulation	2	1	2	2	4
Respiration	110	125	117	121	120
Stress Response	179	191	186	182	185
Metabolism of Aromatic Compounds	48	54	48	50	47
Amino Acids and Derivatives	468	479	482	475	468
Sulfur Metabolism	23	32	30	40	30
Phosphorus Metabolism	60	53	55	52	57
Carbohydrates	416	477	420	368	402

In addition, alignment of the whole genome sequence of the strain NP 3b2^T to the available whole genome sequences of *T. profundimaris* WP0211^T, *T. xiamenensis* M-5^T, *T. permensis* NBRC 106175^T and *T. lucentensis* QMT2^T also provided the average nucleotide identity (ANI) and *in silico* genome-to-genome distance (GGD) for bacterial taxonomic determination. ANI and GGD values were found to be 76-82% and 21-25% (Table 4.5.3), respectively. These values were significantly lower than the threshold value of 95-96% for ANI of members of the same species (Ramasamy et al., 2014, Kim et al., 2014, Richter and Rosselló-Móra, 2009) and 70% for GGD (Thompson et al., 2013).

Table 4.5.3 Comparative genomic characteristics of strain NP 3b2^T and other species of the genus *Thalassospira.*

Organism	**strain NP 3b2^T**			
	DNA-DNA relatedness (%)	**ANI (%)**	**GGD (%)**	**16S rRNA gene (%)**
H 94	80.23	ND*	ND	99.8
T. alkalitolerans JCM 18968^T	55.5	ND	ND	98.0
T. lucentensis DSM 14000^T	61.0	78.6	22.2	95.0
T. mesophila JCM 18969^T	58.5	ND	ND	97.5
T. povalilytica JCM 18746^T	60.5	ND	ND	99.4
T. profundimaris DSM 17430^T	60.5	82.2	25.5	99.3
T. tepidiphila 1-1B^T	53.5	ND	ND	98.7
T. xiamenensis DSM 17429^T	59.0	76.6	21	98.6
T. xianhensis JCM 14850^T	58.5	ND	ND	99.0
'*T. permensis'* NBRC 106175^T	ND	76.6	21.1	99.0
T. lohafexi 139Z-12^T	ND	ND	ND	98.4

*, ND, no data available

The genotype to phenotype analysis was also carried out based on the avalaible whole genome sequence of a species. As seen from the data presented in Table 4.5.4, the results of the physiological and biochemical analyses are consistent when comparing the *in silico* results, however a few discrepancies were noted. A similar level of deviation was previously reported in the case of *Vibrio* and *Marinobacter* species; in those cases it was suggested that expression of certain genes may be restricted by stop codon, repressor genes, regulatory proteins, global regulators, genome coverage or sequencing errors (Amaral et al., 2014, Ng et al., 2014).

Table 4.5.4 Comparative phenotypic characteristics based on *in silico* genomic analysis and *in vitro* physiological and biochemical tests.

Characteristics	**NP 3b2^T**		***T. profundimaris* WP0211^T**		***T. xiamenensis* M-5^T**		***T. lucentensis* QMT2^T**	
	In vitro	***In silico***	***In vitro***	***In silico***	***In vitro***	***In silico***	***In vitro***	***In silico***
Nitrate reduction	+	+	+	+	-	+	-	+
Nitrite reduction	-	+	ND*	+	+	+	-	+
Gelatinase	-	-	-	-	-	-	ND	-
Indole production	-	-	-	-	-	-	-	-
D-Glucose	-	-	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	+	+
Maltose	-	+	-	-	+	+	+	+
D-Mannitol	-	-	+	-	+	-	-	-
Sucrose	-	+	-	-	+	+	-	+
Ornithine	-	+	-	+	+	+	+	+
N-acetyl-β-glucosaminidase	-	-	ND	-	+	-	+	-
Citrate	-	-	-	-	-	-	+	-
Glycerol	-	+	+	+	+	+	+	+

* ND, no data available

4.6 Summary

Based on the results of the phylogenetic and genomic analyses, strains NP 3b2^T and H 94 can be considered as distinct species of the genus *Thalassospira.* This finding was also supported by the comparative analysis of the phenotypic, chemotaxonomic and genetic characteristics including the lower than 70% DNA-DNA hybridization threshold value routinely applied for species differentiation. The two new strains can be readily distinguished from other *Thalassospira* species by a combination of several physiological and biochemical features, *e.g*., the salinity tolerance, nitrate reduction, lack of ability to produce H_2S, indole, lysine, ornithine and arginine or hydrolyse gelatine, agar and ability to utilise a wide range of carbon sources. Strains NP 3b2^T and H 94 shared 99.8% of their 16S rRNA gene sequences with 80.23% of DDH relatedness, supporting the premise that the two strains belong to the same geno-species. On the basis of the results presented in this study, it can be concluded that strains NP 3b2^T and H 94 represent a new species of the genus *Thalassospira*, for which the name *Thalassospira australica* sp. nov. was proposed, with strain NP 3b2^T being the type strain of the genus. The etymology for *Thalassospira australica* is aus.tra'li.ca N.L. fem. adj., australica and the type strain is NP 3b2^T (= KMM 6365^T = JCM 31222^T), isolated from sea water taken from St. Kilda Beach, Port Philip Bay, Victoria, Australia.

Chapter 5.

Computational identification of microRNAs in *Thalassospira* bacteria

5.1 Overview

MicroRNA (miRNA) is a class of small, non-coding RNA molecules containing 19-22 nucleotides. Since the discovery of miRNA in *Caenorhabditis elegans* (Lee et al., 1993), a large number of miRNA molecules have been reported in animals, plants and viruses as key players in regulation of gene expression network (Cullen, 2006, Takeda and Watanabe, 2006, Gottesman, 2005, Plasterk, 2006). In bacteria, the small non-coding RNA (sRNA) have been demonstrated to have a similar function to eukaryotic miRNA in modulating the target mRNA in various ways at a post-transcriptional level (Gottesman and Storz, 2011) (as discussed in Chapter 2). A number of sRNA has been identified in bacteria, some of which were identified in marine bacteria such as *Vibrionaceae* and *Synechococcus*. These have functional analogues to plant miRNAs in response to environmental changes (Gierga et al., 2012, Nguyen and Jacq, 2014). The investigations of bacterial miRNAs have gained little attention, except for the recent studies on miRNAs derived from *Streptococus mutants* ATCC 25175 and *Escherichia coli* DH10B (Lee and Hong, 2012, Kang et al., 2013).

The genus *Thalassospira* includes Gram-negative, aerobic and halophilic bacteria dwelling in a marine environment (Shivaji et al., 2015). Bacteria of this genus are involved in the biodegradation of a variety of hydrocarbons (Kodama et al., 2008, Nogi et al., 2014, Ivanova et al., 2016). For example, *T. tepidiphila* 1-1B^T and *T. povalilytica Zumi* 95^T have the ability to degrade polycyclic aromatic hydrocarbons and polyvinyl alcohol (Kodama et al., 2008, Nogi et al., 2014); and *T. australica* NP 3b2^T is able to utilise poly (ethylene) terephthalate (PET) plastic as a carbon source (Ivanova et al., 2016). Currently the genus *Thalassospira* is comprised of 10 validly named species, of which the whole genome sequences of four species (*T. australica* NP 3b2^T*, T. lucentensis* QMT2^T, *T. profundimaris* WP0211^T and *T. xiamenensis* M-5^T) have been assembled and deposited in public databases (Lai and Shao, 2012b, Lai and Shao, 2012a, López-Pérez et al., 2014). The presence of miRNAs in bacteria of the genus has, however, not been demonstrated. In this chapter, the investigation of miRNA populations was carried out using 9 validly named *Thalassospira* species, including *T. alkalitolerans* JCM 18968^T, *T. lucentensis* QMT2^T, *T. mesophila* JCM 18969^T, *T. povalilytica* Zumi 95^T, *T. profundimaris* WP0211^T, *T. tepidiphila* 1-1B^T, *T. xiamenensis* M-5^T, *T. xianhensis* P-4^T and *T. australica* NP 3b2^T. Since *Thalassospira lohafexi* 139Z-12^T was not published until 2015 (Shivaji et al., 2015), this species was not included in this study. A small RNA-

Seq approach was used to identify the bacterial miRNAs (Howard et al., 2013). Thus, the aim of this work was to identify the potential miRNAs in bacteria of the genus *Thalassospira* using computational approaches from small RNA sequence dataset generated by high-throughput sequencing technology.

5.2 Quality of total RNA extraction

A bacterial small RNA-seq was generated using the RNA isolated from the bacterial cells of nine *Thalassospira* species. The total RNA was extracted from cultures after 2 days of incubation at 25°C using the TRIsure reagent for cell lysis, as detailed in Chapter 3. After extraction, total RNA was checked for its integrity and quality using a Bioanalyzer (Agilent Technologies, USA). The RNA was further used for the preparation of a small RNA library if two distinct ribosomal RNA peaks, representing the 16S and 23S for prokaryotic RNA, were identified in the electropherogram (Figure 5.2.1).

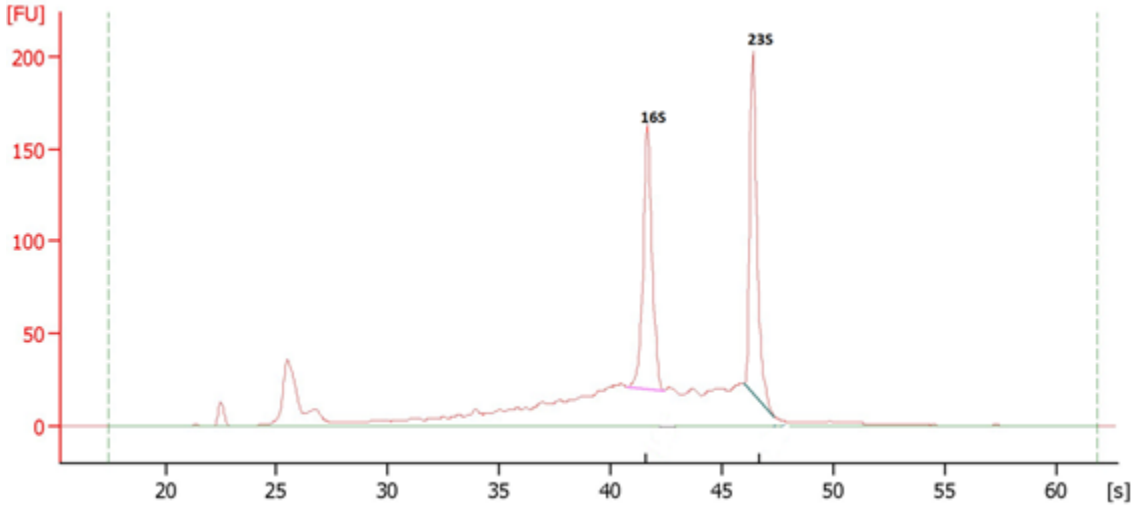

Figure 5.2.1 Example of an electropherogram for testing the quality of total bacterial RNA of *T. alkalitolerans* JCM 18968^T used for small RNA-seq. Total RNA was isolated from cells and evaluated using an Agilent 2100 Bioanalyzer.

5.3 Generation of small RNA libraries and data evaluation

After passing the quality control test for RNA, small RNA-seq libraries were constructed for nine *Thalassospira* species, including *T. alkalitolerans* JCM 18968^T, *T. lucentensis* QMT2^T, *T. mesophila* JCM 18969^T, *T. povalilytica* Zumi 95^T, *T.*

profundimaris WP0211^T, *T. tepidiphila* 1-1B^T, *T. xiamenensis* M-5^T, *T. xianhensis* P-4^T and *T. australica* NP 3b2^T. The next-generation small RNA-seq was used for detection of the presence of small RNAs. The results yielded 242,204,737 sequence reads of 18–33 nucleotides (nt) in length, with 22,991,595 from *T. australica* NP 3b2^T, 31,431,409 from *T. alkalitolerans* JCM 18968^T, 28,737,289 from *T. lucentensis* QMT2^T, 27,213,115 from *T. mesophila* JCM 18969^T, 28,225,762 from *T. povalilytica* Zumi 95^T, 25,272,618 from *T. profundimaris* WP0211^T, 25,641,708 from *T. tepidiphila* 1-1B^T, 28,144,650 from *T. xiamenensis* M-5^T and 24,546,591 from *T. xianhensis* P-4^T (Table 5.3.1). The reads were curated for any contamination or artefacts such as rDNA or Illumina small RNA adaptor sequences using Cutadapt (Martin, 2011). These sequences were then collapsed to eliminate any repeated sequences before alignment. The collapsed reads were then aligned to the *Thalassospira* genome sequences available in the NCBI database using Bowtie software (Langmead et al., 2009) with default setting.

Since the whole genome sequences of *T. australica* NP 3b2^T, *T. lucentensis* QMT2^T, *T. profundimaris* WP0211^T and *T. xiamenensis* M-5^T were available in the NCBI database, the small RNA sequence reads were aligned to these genome sequences. The remaining 5 *Thalassospira* species that did not have the whole genome sequence were aligned to these references based on their phylogenetically closest species (Ivanova et al., 2016) (Table 5.3.2). By aligning 3.17, 5.06, 4.60, 2.95, 3.76, 5.32, 4.36, 1.71 and 3.30 million collapsed reads from *T. australica* NP 3b2^T, *T. alkalitolerans* JCM 18968^T, *T. lucentensis* QMT2^T, *T. mesophila* JCM 18969^T, *T. povalilytica* Zumi 95^T, *T. profundimaris* WP0211^T, *T. tepidiphila* 1-1B^T, *T. xiamenensis* M-5^T and *T. xianhensis* P-4^T, respectively, to the genome sequences available in NCBI using Bowtie software (Langmead et al., 2009), it was found that a proportion of reads with 2.28 million from *T. australica* NP 3b2^T, 1.60 million from *T. alkalitolerans* JCM 18968^T, 2.51 million from *T. lucentensis* QMT2^T, 102,497 from *T. mesophila* JCM 18969^T, 95,972 from *T. povalilytica* Zumi 95^T and 2.04 million, 1.23 million, 1.14 million and 631,902 from *T. profundimaris* WP0211^T, *T. tepidiphila* 1-1B^T, *T. xiamenensis* M-5^T and *T. xianhensis* P-4^T libraries, respectively, were mappable to the reference genomes, accounting for 71.94% of *T. australica* NP 3b2^T, 3.18% of *T. alkalitolerans* JCM 18968^T, 54.53% of *T. lucentensis* QMT2^T, 3.47% of *T. mesophila* JCM 18969^T, 2.55% of *T. povalilytica* Zumi 95^T, 38.42% of *T. profundimaris* WP0211^T, 28.16% of *T. tepidiphila* 1-1B^T, 66.76% of

T. xiamenensis M-5^{T} and 19.14% of *T. xianhensis* P-4^{T} (Table 5.3.1). The aligned reads were further analysed for miRNA identification.

Table 5.3.1 Bacterial small RNA-Seq data yield from Hiseq2500 sequencing.

Samples	***T. australica* NP 3b2^T**	***T. alkalitolerans* JCM 18968^T**	***T. lucentensis* QMT2^T**	***T. mesophila* JCM 18969^T**	***T. povalilytica* Zumi 95^T**	***T. profundimaris* WP0211^T**	***T. tepidiphila* 1-1B^T**	***T. xiamenensis* M-5^T**	***T. xianhensis* P-4^T**
Original number of reads (million)[a]	22,991,595	31,431,409	28,737,289	27,213,115	28,225,762	25,272,618	25,641,708	28,144,650	24,546,591
Number of reads after collapse (million)[b]	3,177,403	5,068,260	4,606,730	2,954,668	3,761,719	5,327,201	4,368,445	1,712,391	3,300,999
Number of reads aligning to reference genome (million)[c]	2,285,671	160,990	2,511,917	102,497	95,972	2,046,567	1,230,299	1,143,173	631,902
% reads aligned	71.94	3.18	54.53	3.47	2.55	38.42	28.16	66.76	19.14

[a]: total reads obtained from different bacterial species; [b]: reads obtained after filtering; [c]: reads were aligned to the *Thalassopsira* genome sequences using the Bowtie algorithm using the default settings. The reads obtained from this experiment were set from 18-33 nt using an Illumina genome analyser with a low error rate (0.005 error per base).

Table 5.3.2 Whole genome sequences of bacteria of the genus *Thalassospira* available in GenBank.

Samples	References	GenBank accession numbers
T. australica NP 3b2^T	*T. australica* NP 3b2^T	JRJE00000000.1
T. lucentensis QMT2^T	*T. lucentensis* QMT2^T	ATWN00000000.1
T. alkalitolerans JCM 18968^T		
T. mesophila JCM 18969^T		
T. profundimaris WP0211^T	*T. profundimaris* WP0211^T	AMRN00000000.1
T. tepidiphila 1-1B^T		
T. povalilytica Zumi 95^T		
T. xiamenensis M-5^T	*T. xiamenensis* M-5^T	CP004388
T. xianhensis P-4^T		

5.4 Identification of miRNAs using the Mirdeep2 method

Identification of bacterial miRNAs is in the early stages in comparison to animal and plants. Therefore, no specific method has been developed for investigation of miRNAs in bacteria. miRDeep2 software (http://www.mdc-berlin.de/rajewsky/miRDeep) was first presented as an algorithm to predict miRNA in human and animals (Friedlander et al., 2008). This software was later used to successfully identify miRNAs in plant (Hackenberg et al., 2013a). Based on the applicability in different organisms, miRDeep2 software was, therefore, employed to detect putative bacterial miRNAs from the millions of short sequences generated from the dataset. The miRDeep2 software was designed to detect miRNAs based on an investigation of the secondary structure of the miRNA precursor sequences. The potential miRNA precursors were then integrated with a model for miRNA precursor processing using Dicer, releasing the mature miRNA sequences, star sequences (or called miRNA5p and miRNA3p sequences) and the loop (Friedlander et al., 2008). By using miRDeep, 86 potential miRNA precursor sequences could be identified from the dataset. The putative mature

miRNA, star sequence and the loop were also identified within each precursor (Figure 5.4.1).

The identified 86 putative mature miRNAs were located in either the 5' arm or the 3' arm of the precursors, with 11 sequences from *T. australica* NP 3b2^T, 11 sequences from *T. lucentensis* QMT2^T, 14 sequences from *T. profundimaris* WP0211^T, 6 sequences from *T. xiamenensis* M-5^T, 11 sequences from *T. alkalitolerans* JCM 18968^T, 5 sequences from *T. mesophila* JCM 18969^T, 4 sequences from *T. povalilytica* Zumi 95^T, 9 sequences from *T. tepidiphila* 1-1B^T and 15 sequences from *T. xianhensis* P-4^T (Table 5.4.1) being obtained.

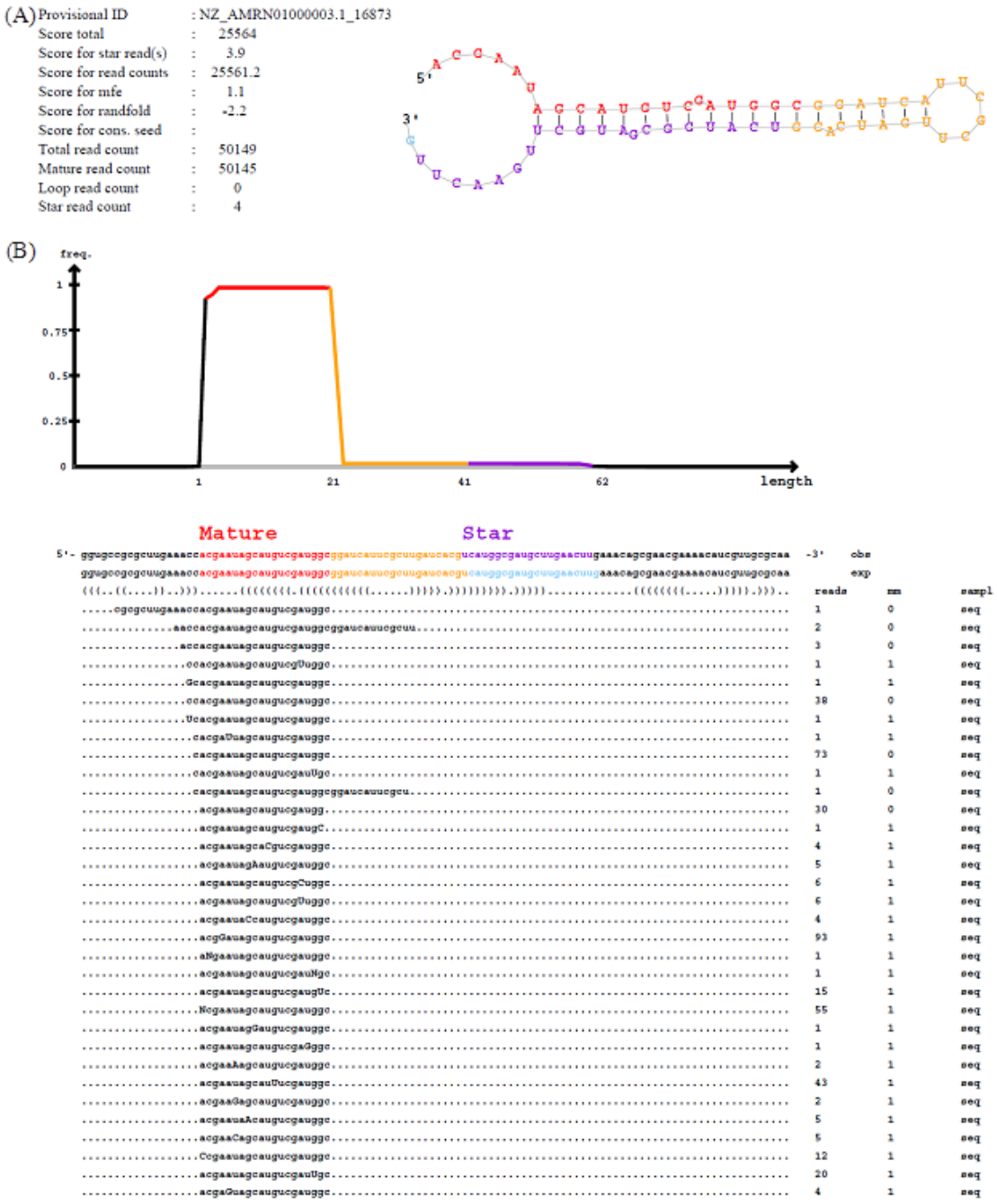

Figure 5.4.1 An example of miRDeep output for T.prof_5p_16873. Part (A): predicted secondary structure of miRNA precursor with the mature, star and loop sequences (highlighted in red, yellow and purple, respectively). The scores associated to the miRNA, the read counts for mature, star and loop sequences and the total read count are also given. Part (B): sequences of the predicted miRNA precursors mapped to the mature, star and loop sequences on the genome (obs line) and the experimental sequence reported in miRBase (exp line). The frequency (reads column) and mismatches of the read with the genomic sequence (mm column) are also given with the mismatches shown in capital letters.

Table 5.4.1 The putative miRNAs of bacteria of the genus *Thalassospira* identified using miRDeep2.

miRNA name*	Location	Sequence (5'-3')	Length (nucleotides)	Number of reads
***T. australica* NP 3b2^T**				
T.aust_3p_10018	JRJE01000008.1_scaffold_30_10018	TAACGTCTGTCCTTCGGATT	20	2252
T.aust_5p_26441	JRJE01000032.1_scaffold_0_26441	CTTGGCAGGCTGGGCGCTCC	20	845
T.aust_3p_11556	JRJE01000009.1_scaffold_3_11556	TTTTGACTGGATCGGCAACCGTGAT	25	151
T.aust_5p_26002	JRJE01000032.1_scaffold_0_26002	TTTGGCGGGGTCGGGAACC	19	88
T.aust_5p_15617	JRJE01000022.1_scaffold_18_15617	ATCCTCTCCCCGCAACCA	18	2888
T.aust_5p_9704	JRJE01000008.1_scaffold_30_9704	ATTGGCGTCACAGATCAGGGGCAT	24	15
T.aust_3p_23062	JRJE01000031.1_scaffold_1_23062	GAAATCCCTGATCGCGCAG	19	13
T.aust_5p_10770	JRJE01000009.1_scaffold_3_10770	ACAAATCTCGGCAAGGCC	18	229
T.aust_5p_13346	JRJE01000019.1_scaffold_20_13346	CGAACTCTGCACCAAGGC	18	12
T.aust_5p_22895	JRJE01000031.1_scaffold_1_22895	CGAATCTCTCATCACCCACCA	21	4199
T.aust_5p _5988	JRJE01000005.1_scaffold_5_5988	AAACCGGATCCTGCAGCC	18	9
***T. alkalitolerans* JCM 18968^T**				
T.alka_5p_5662	ATWN01000006.1_5662	TCACCGGTTGGGAAGGCGCTGA	22	15
T.alka_3p_6962	ATWN01000007.1_6962	CTTCCCGCCCCATGGCCGA	19	14
T.alka_3p_6323	ATWN01000007.1_6323	AATTAATGGGTCCTGACC	18	336
T.alka_5p_4136	ATWN01000004.1_4136	TTTCGGGTGGGCAGCGCC	18	1

miRNA name*	Location	Sequence (5'-3')	Length (nucleotides)	Number of reads
T.alka_3p_1438	ATWN01000001.1_1438	CGCATGGGCGGAGCTTTTCGTTAG	24	13
T.alka_3p_6324	ATWN01000007.1_6324	AATTAATGGGTCCTGACC	18	336
T.alka_3p_2121	ATWN01000002.1_2121	ATTGATTGCGGCCATCCG	18	11
T.alka_5p_4462	ATWN01000005.1_4462	CAAGAACCGCCATCTGCATGCC	22	9
T.alka_5p_1889	ATWN01000002.1_1889	ATGCTTTTTGGCCGCATT	18	1
T.alka_3p_75	ATWN01000001.1_75	CGAGGTCGAACATGATGAA	19	25
T.alka_5p_3621	ATWN01000003.1_3621	ATGTTGCCGGTGCGGCGGCGGGC	23	7
***T. lucentensis* QMT2^{T}**				
T.luce_5p_27853	ATWN01000011.1_27853	CCGAGGTCCGGTATCGCCTGACT	23	13160
T.luce_5p_31831	ATWN01000015.1_31831	ATCGTGGCCGCACTGGAGCC	20	907
T.luce_3p_14305	ATWN01000004.1_14305	CGCGCAGGCGGGGATCTCGAGC	22	1860
T.luce_5p_11956	ATWN01000003.1_11956	ATCGCTGCGGGCAATAAAAGACC	23	60
T.luce_5p _2660	ATWN01000001.1_2660	TTACCCGTGAGGTCGGCTGTGCGAT	25	163
T.luce_5p_10500	ATWN01000003.1_10500	ATAATGACGTCCGTTGCGAC	20	922
T.luce_3p_13875	ATWN01000004.1_13875	AAACGGGGTCGGGGGGCTG	19	3395
T.luce_3p_12006	ATWN01000003.1_12006	ACCACAGGTGCGGGCATGGGCATG	24	158
T.luce_5p_14218	ATWN01000004.1_14218	AAAGCCCCGGCGCGATTGTCC	21	158
T.luce_3p_14713	ATWN01000004.1_14713	TTTGCGCGATGGGTCCCTGAT	21	17
T.luce_3p_22524	ATWN01000007.1_22524	TCACAGTCGAGACGCTCTCTCACC	24	50057

miRNA name*	Location	Sequence (5'-3')	Length (nucleotides)	Number of reads
***T. mesophila* JCM 18969^T**				
T.meso_5p_3464	ATWN01000007.1_3464	ATAAGGAGTAGGCGAATGAGC	21	69
T.meso_3p_3562	ATWN01000007.1_3562	TCACAGTCGAGACGCTCTCTCACC	24	10
T.meso_3p_3026	ATWN01000006.1_3026	CTTGGCGTCGAAGGCATGA	19	2
T.meso_5p_2868	ATWN01000006.1_2868	TTTGGCAAGGCACAGCGCGCAG	22	9
T.meso_3p_3086	ATWN01000006.1_3086	CTGCGCGCTGTGCCTTGCC	19	9
***T. povalilytica* Zumi 95^T**				
T.pova_5p_4964	AMRN01000014.1_4964	ATCTTTCGATGGTCGTGGCA	20	251
T.pova_5p_4720	AMRN01000012.1_4720	CCAAGCGCGGTGCGGACCG	19	21
T.pova_5p_661	AMRN01000001.1_661	ATGGGCATCCTGACCGAAGGCACG	24	8
T.pova_5p_390	AMRN01000001.1_390	CTTGAAGACCTGCATCAGCGTTC	23	7
***T. profundimaris* WP0211^T**				
T.prof_5p_16873	AMRN01000003.1_16873	ACGAATAGCATGTCGATGGC	20	50145
T.prof_5p_35057	AMRN01000009.1_35057	ATCGCCTGAACGCGCGCCTGACCG	24	1316
T.prof_5p_36012	AMRN01000010.1_36012	GTCCGGTGGTCTGGGCACCATG	22	1679
T.prof_5p_14768	AMRN01000003.1_14768	ATCCTGCCCCCGCAACCA	18	721
T.prof_5p_660	AMRN01000001.1_660	CTATGCAGACACCCCGGAC	19	1702
T.prof_5p_26017	AMRN01000006.1_26017	ATCACGTTGAGCCAAAAGAAAAGC	24	18
T.prof_5p_15266	AMRN01000003.1_15266	ATACAACTGATGTCGCCTGC	20	693

miRNA name*	Location	Sequence (5'-3')	Length (nucleotides)	Number of reads
T.prof_3p_2147	AMRN01000001.1_2147	ATCCTCGGAATAGGTATAGGCTTCC	25	57
T.prof_3p_20338	AMRN01000004.1_20338	ATCAATCGCCGGGATCATGATCCC	24	76
T.prof_5p_12543	AMRN01000002.1_12543	ATATACGGCCTGGCATAATC	20	2
T.prof_5p_2228	AMRN01000001.1_2228	TTTGCGGAATGCCACCCGGCAACG	24	10
T.prof_5p_19418	5' AMRN01000004.1_19418	GTGTTCTTTTGGTCGCGCATGCCG	24	11
T.prof_3p_6436	AMRN01000001.1_6436	AAAAGACCGTCCTGCCACCG	20	6
T.prof_3p_49	AMRN01000001.1_49	CTCCTGAGCCGGGCCAAT	18	9
***T. tepidiphila* 1-1B^{T}**				
T.tepi_5p_8152	AMRN01000002.1_8152	ATAATGACGTCCGTTGCGA	19	90
T.tepi_5p_16607	AMRN01000006.1_16607	TTCAAGTCTGATGCCCGCGCC	21	9
T.tepi_5p_22622	AMRN01000010.1_22622	GTCCGGTGGTCTGGGCACCATG	22	252
T.tepi_5p_2762	AMRN01000001.1_2762	GCAGTGGCTTGGCGGGATCGGGAT	24	148
T.tepi_5p_19992	AMRN01000008.1_19992	GGGCCGAGATCGAAAGCAACACG	23	17
T.tepi_5p_8570	AMRN01000003.1_8570	ATCCTGCCCCCGCAACCA	18	1135
T.tepi_5p_11638	AMRN01000004.1_11638	TTTGTCGTTCTGGGCTGGCA	20	12
T.tepi_5p_21088	AMRN01000009.1_21088	ATTGATATCGCATCGGTTACCGA	23	65
T.tepi_5p_23416	AMRN01000011.1_23416	GTATATTGCCAATTTTGT	18	88
***T. xiamenensis* M-5^{T}**				
T.xiam_3p_12910	CP004388.1_12910	CTTGCCGCCGGTATGCTCGCATC	23	2582

miRNA name*	Location	Sequence (5'-3')	Length (nucleotides)	Number of reads
T.xiam_5p_18209	CP004388.1_18209	ATGCAGATCGGTTTGCGCACC	21	47
T.xiam_5p_1950	CP004388.1_1950	ACGGTTTGCGTCGGTCACGCTGGC	24	331
T.xiam_5p_12633	CP004388.1_12633	ATGGACTCCCGCTTTCGC	18	12
T.xiam_5p_12105	CP004388.1_12105	ATTTGCATGCCCGTCTGGC	19	13
T.xiam_3p_3097	CP004388.1_3097	AGCATTCAAGCATCGGCGGGAT	22	13
***T. xianhensis* P-4^{T}**				
T.xian_5p_4477	CP004388.1_4477	TAGGCGGGAGTCCACCGGGC	20	5
T.xian_3p_19710	CP004388.1_19710	GGATCAGCTGGGTAACATC	19	11
T.xian_3p_20546	CP004388.1_20546	CTTGCACCGGGCCGCTTTCGGATG	24	14
T.xian_3p_19849	CP004388.1_19849	ACGCGACCGCGGCAAGGAAA	20	24
T.xian_5p_25207	CP004388.1_25207	AAAGCAGGAAGAATACGAACAGA	23	27
T.xian_5p_31617	CP004388.1_31617	ATGCACCCGGACCGAAACCC	20	558
T.xian_3p_3269	CP004388.1_3269	AAAGCGCGCCCCCTTGCTCCC	21	43
T.xian_5p_9480	CP004388.1_9480	ATTCAGGAATCTGTTCTGACGCAGC	25	26
T.xian_5p_19360	CP004388.1_19360	ATTTTAGTCCGCGTCGCAAC	20	13
T.xian_3p_38616	CP004388.1_38616	CAAACAGCTGAAGGCCTCCC	20	166
T.xian_3p_35228	CP004388.1_35228	ATTCCGATGATCTGGTGATTG	21	11
T.xian_3p_13589	CP004388.1_13589	TTGCCGATCATCGCCCTTGCCCTG	24	32
T.xian_5p_34659	CP004388.1_34659	CTTCAGTTCCTCGACCTT	18	13

miRNA name*	Location	Sequence (5'-3')	Length (nucleotides)	Number of reads
T.xian_3p_4479	CP004388.1_4479	CTGACTGGATTCCCGCGT	18	9
T.xian_3p_32022	CP004388.1_32022	ATCATGCCGGGCAGATCA	18	8

*: The identified putative miRNAs were named as <name of bacteria>_<location of miRNA in the precursor>_<position of miRNA in the genome>.

As can be seen in the data presented in Table 5.4.1, the identified putative miRNAs are 18 – 25 nucleotides in length, which is in a range of size distribution of the miRNAs previously reported for animals and plants (Millar and Waterhouse, 2005). These results are also consistent with the length distribution of miRNAs recently detected in *E. coli* DH10B and *Streptococcus mutans* ATCC 25175 (Kang et al., 2013, Lee and Hong, 2012). Based on the number of read counts from high through-put sequencing, these potential miRNAs have various expressions among nine libraries, ranging from one to thousands of reads in each library. Among the 86 detected potential miRNAs, the highest expression was found in T.prof_5p_16873 with 50145 reads while the lowest expression was shared between T.alka_5p_4136 and T.alka_5p_1889 with only one read being found in the libraries. Of these putative miRNAs, thirteen were in high abundance, with over a thousand reads (Kang et al., 2013) with three miRNAs from *T. australica* NP 3b2^T, four from *T. lucentensis* QMT2^T, four from *T. profundimaris* WP0211^T and one each from *T. tepidiphila* 1-1B^T and *T. xiamenensis* M-5^T being identified. The high degree of expression of these putative miRNAs suggests that they may play specific role in the growth and developmental processes of these bacteria (Figure 5.4.2).

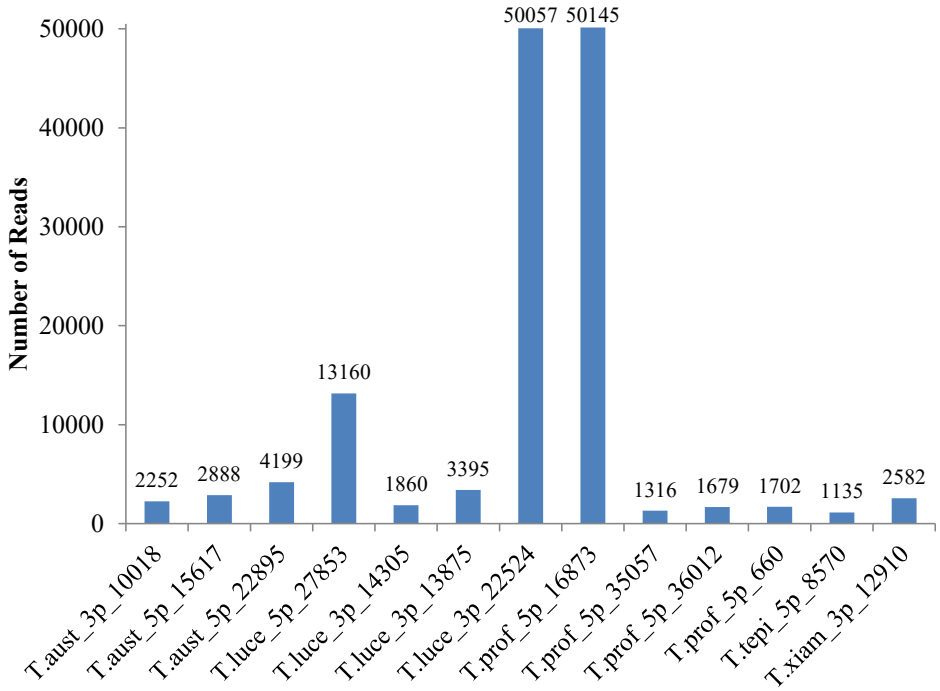

Figure 5.4.2 Expression of miRNAs detected in bacteria of the genus *Thalassospira*. The high degree of expression of miRNAs was found in *T. australica* NP 3b2^T, *T. lucentensis* QMT2^T, *T. profundimaris* WP0211^T, *T. tepidiphila* 1-1B^T and *T. xiamenensis* M-5^T.

5.5 Identification of miRNAs using CID-miRNA method

The 86 putative miRNAs detected in nine *Thalassospira* species using miRDeep method appeared to be rather low compared to 400 miRNAs identified in *E. coli* DH10B (Kang et al., 2013) and 900 miRNAs in *Streptococcus mutans* ATCC 25175 (Lee and Hong, 2012). Therefore, an alternative method, CID-miRNA (http://mirna.jnu.ac.in/cidmirna/) was employed in the expectation of identifying further potential miRNAs from the dataset. CID-miRNA is a web-sever developed for the identification of the miRNA precursors based on the secondary structure filter and an algorithm of stochastic context free grammar (SCFG) (Tyagi et al., 2008). The sever was, firstly, used to predicted the potential miRNA precursors in human genome (Tyagi et al., 2008) which later on applied in animals such as mouse, zebrafish and sea squirt (Tempel and Tahi, 2012).Using the CID-miRNA method, 449 potential miRNA precursors were identified from over 242 million reads of *T. australica* NP 3b2^T (77 sequences), *T. lucentensis* QMT2^T (161 sequences), *T. profundimaris* WP0211^T (78 sequences), *T. xiamenensis* M-5^T (16 sequences), *T. alkalitolerans* JCM 18968^T (21 sequences), *T. mesophila* JCM 18969^T (1 sequence), *T. povalilytica* Zumi 95^T (2 sequences), *T. tepidiphila* 1-1B^T (61 sequences) and *T. xianhensis* P-4^T (32 sequences).

In order to identify the mature miRNAs, these precursor sequences were further analysed by employing another web tool, *MatureBayes* (http://mirna.imbb.forth.gr/MatureBayes.html). *MatureBayes* is designed in incorporation to the Naïve Bayes classifier for identifying the mature miRNA molecules based on the sequence and structure of the miRNA precursors (Gkirtzou et al., 2010). This tool provided mature miRNA position in the 5' and 3' direction of the miRNA precursors, resulting in 898 putative mature miRNAs, which were identified in 9 *Thalassospira* species, including *T. australica* NP 3b2^T (154 putative miRNAs), *T. lucentensis* QMT2^T (322 putative miRNAs), *T. profundimaris* WP0211^T (156 putative miRNAs), *T. xiamenensis* M-5^T (32 putative miRNAs), *T. alkalitolerans* JCM 18968^T (42 putative miRNAs), *T. mesophila* JCM 18969^T (2 putative miRNAs), *T. povalilytica* Zumi 95^T (4 putative miRNAs), *T. tepidiphila* 1-1B^T (122 putative miRNAs) and *T. xianhensis* P-4^T (64 putative miRNAs) (Appendix 1). These identified putative miRNAs were used for further analysis of miRNA conservation.

5.6 Comparison of putative bacterial miRNAs identified using both the miRDeep2 and CID-miRNA methods

miRDeep2 is a robust tool used for the detection of high-confidence miRNA candidates in the genome (Friedlander et al., 2008). The algorithm can detect very low abundance levels of miRNAs. Due to excessive false-positives, which exceed the allowed cut-off value, the detection of miRNA is limited (Friedlander et al., 2008). For this reason, only 86 putative miRNAs were detected by miRDeep2 from over 242 million reads of 9 species. The use of CID-miRNA tool for identifying miRNAs obtained an overall higher number of miRNA detection in the dataset. The CID-miRNA tool, however, only identifies the sequences and secondary structures of the miRNA precursors. Thus, these need further analysis for the identification of mature miRNAs using another bioinformatics tool. By using CID-miRNA analysis, 499 potential miRNA precursors were detected in 9 small RNA libraries of 9 *Thalassospira* species, resulting in the identification of 898 putative mature miRNA sequences using *MatureBayes*. Therefore, it is suggested that the data identified by both methods be used for further analysis. In order to determine whether miRNAs were detected using miRDeep2 and also identified by using CID-miRNA, miRNA data generated from miRDeep2 was blasted to miRNA data produced by CID-miRNA. The results showed that there were no shared putative mature miRNA sequences. miRDeep2 predicts putative miRNAs based on distribution of minimum free energy (MFE) and stability of secondary structure established for nematode, *C. elegans*, while CID-miRNA utilised secondary structure filter and stochastic context free grammar trained on human miRNAs (Friedlander et al., 2008; Tyagi et al., 2008). The differences of parameter setting may cause no overlap of putative mature miRNA sequences detected in both methods. Thakur et al. (2011) also pointed out

that application of new parameters improved the accuracy of plant miRNA prediction using miRDeep in compared to the default setting for animals.

Study on miRNA biogenetic showed that one or more mature miRNAs can be produced from one pre-miRNA molecule (Bologna et al., 2013). These identified putative miRNAs, therefore, were blasted to the potential precursor miRNA sequences generated by both methods. Putative mature miRNA sequences identified from miRDeep2 were blasted to the potential precursor miRNAs detected by CID-miRNA and vice versa. 5 putative mature miRNA sequences identified by CID-miRNA (T.luce_5p_228121, T.luce_3p_228121, T.xian_5p_2738, T.xian_5p_2740 and T.xian_3p_2740) were found to locate in 3 potential precursor miRNAs producing T.luce_5p_11956, T.xian_5p_4477 and T.xian_3p_4479 from miRDeep2. The precursor miRNA sequences producing 5 putative mature miRNAs from CID-miRNA were then aligned to 3 from miRDeep2 using CLUSTALW. The result showed the overlap of 3 precursor miRNAs predicted in both methods in which the putative mature miRNAs identified by each method located at different positions within the precursor sequences (Figure 5.6.1).

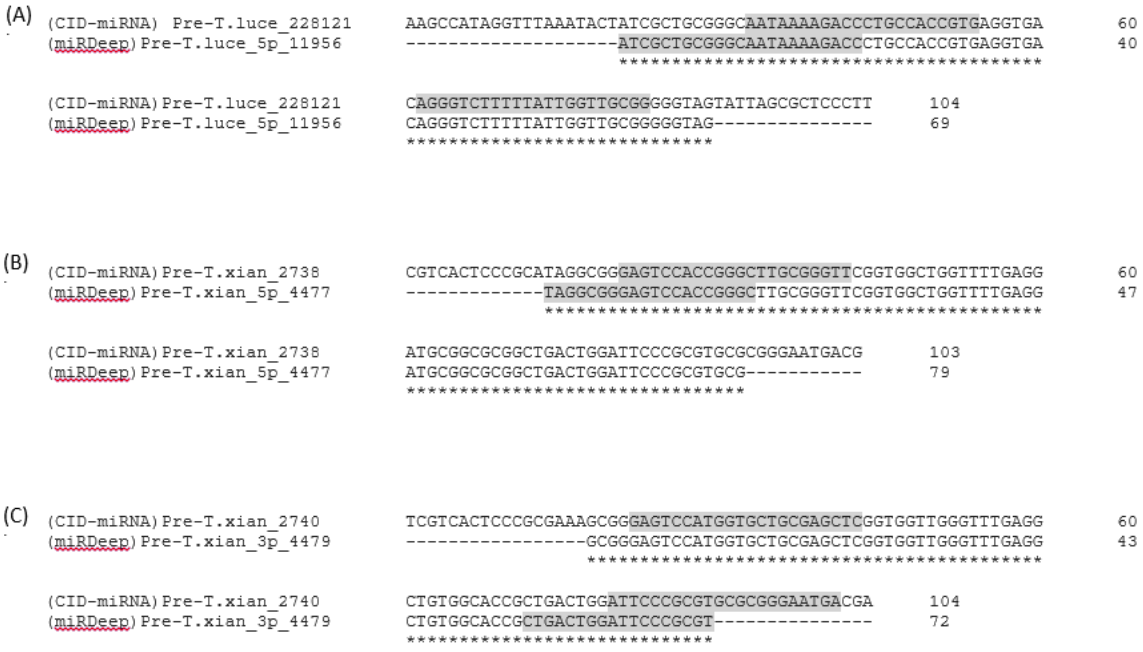

Figure 5.6.1 Sharing sequences of potential miRNA precursors identified in both miRDeep2 and CID-miRNA methods. **(A)** T.luce_228121 precursor from CID-miRNA and T.luce_5p_11956 precursor from miRDeep2. **(B)** T.xian_2738 precursor from CID-miRNA and T.xian_5p_4477 precursor from miRDeep2. **(C)** T.xian_2740 precursor from CID-miRNA and T.xian_3p_4479 precursor from miRDeep2. The asterisk (*) indicate that the nucleotides are identical to the top sequence. The putative mature miRNAs are highlighted in grey. (CID-miRNA) indicated the potential precursor detected by CID-miRNA method. (miRDeep2) indicated the potential precursor detected by miRDeep2 method.

The shared sequences from the same *Thalassospira* species proved the probability of putative miRNAs finding in both methods. Thus, a total of 984 putative miRNA candidates were preliminarily obtained from both methods with default setting together for 9 species (Table 5.6.1). Further work, such as real time PCR or Northern blot analysis, is needed to verify these putative miRNAs.

Table 5.6.1 Identification of putative miRNAs using miRDeep and CID-miRNA methods.

Organism	miRDeep analysis	CID-miRNA analysis	Total
***T. alkalitolerans* JCM 18968^T**	11	42	53
***T. australica* NP 3b2^T**	11	154	165
***T. lucentensis* QMT2^T**	11	322	333
***T. mesophila* JCM 18969^T**	5	2	7
***T. povalilytica* Zumi 95^T**	4	4	8
***T. profundimaris* WP0211^T**	14	156	170
***T. tepidiphila* 1-1B^T**	9	122	131
***T. xianhensis* P-4^T**	15	64	79
***T. xiamenensis* M-5^T**	6	32	38

5.7 Conservation of putative miRNAs in the *Thalassospira* genus and miRNAs previously described in *Escherichia coli* and *Streptococcus mutans*

Previous studies showed the conservation of some miRNAs across animals or the plant kingdom (Wheeler et al., 2009, Schreiber et al., 2011); however, novel sequences can only be found in a particular species. The conserved and novel miRNAs in bacteria still remain unknown, in comparison to intensive studies of miRNAs in eukaryotic organisms and viruses. In this study, 984 putative miRNA candidates of the genus *Thalassospira* were blasted against those reported for *E. coli* DH10B (400 miRNAs) and *S. mutans* ATCC 25175 (900 miRNAs) (Lee and Hong, 2012, Kang et al., 2013) in order to identify any conserved miRNA. However, no conserved sequences could be found without any mismatch or with three and fewer nucleotide substitution. *E. coli* is a enteric bacterium that commonly found in low intestine of humans and animals (Kaper et al., 2004), *S. mutans* is an oral pathogen that causes human dental caries (Ajdić et al., 2002), while bacteria in the *Thalassospira* genus are environmental bacteria (Ivanova et al., 2016). The differences in their characteristics may influence the low extent of conservation of the miRNAs among these bacteria. The results obtained in this study is in agreement with previous studies, that reported the lack of significant sequence similarity in non-coding RNA homologues of different bacterial species (Zhao et al., 2010b, Livny and Waldor, 2007).

In order to identify any conserved putative miRNAs among the bacteria of the genus *Thalassospira*, the putative sequences that were obtained were compared. It appears that the bacteria of the nine studied species of the genus shared 57 common putative miRNAs. Among these, *T. profundimaris* WP0211^T and *T. tepidiphila* 1-1B^T had the highest number of conserved putative miRNAs, with 45 sequences presenting in both species. Five putative miRNA sequences in *T. alkalitolerans* JCM 18968^T were also found in the *T. lucentensis* QMT2^T, while *T. xianhensis* P-4^T and *T. xiamenensis* M-5^T shared 4 conserved sequences. One sequence was found to be shared between *T. australica* NP 3b2^T and *T. tepidiphila* 1-1B^T, *T. lucentensis* QMT2^T and *T. tepidiphila* 1-1B^T and *T. lucentensis* QMT2^T and *T. mesophila* JCM 18969^T (Table 5.7.1). As seen from the data, *T. lucentensis* QMT2^T have identical putative miRNA sequences as *T. alkalitolerans* JCM 18968^T (5 sequences), *T. tepidiphila* 1-1B^T (1) and *T. mesophila* JCM 18969^T (1), while *T. tepidiphila* 1-1B^T also shared conserved sequences with *T. profundimaris* WP0211^T (45) and *T. australica* NP 3b2^T (1).

A comparative analysis of 16S rRNA sequence similarities revealed that the highest number of common putative miRNAs, identified in similar location of their genomes, was shared by the phylogenetically close species *e.g.*, *T. tepidiphila* 1-1B^T was found to be phylogenetically closely related to *T. profundimaris* WP0211^T (99.3% sequence similarity); *T. xianhensis* P-4^T and *T. xiamenensis* M-5^T also shared 99.3% 16S rRNA similarity, while *T. mesophila* JCM 18969^T and *T. alkalitolerans* JCM 18968^T shared 95.3% and 94.9% of 16S rRNA sequence similarity, respectively, with *T. lucentensis* QMT2^T (Ivanova et al., 2016). Conserved putative miRNA sequences found in these species may indicate a close genetic relationship and that these putative miRNAs have a similar role in the regulation of the growth and development of bacteria. It is interesting to note that two species, *T. australica* NP 3b2^T and *T. tepidiphila* 1-1B^T, which are capable of hydrocarbon degradation (Ivanova et al., 2016, Kodama et al., 2008), shared one conserved putative miRNAs, suggesting it might play a role in the regulation of hydrocarbon degradation. However, potential targets of this putative miRNA needs to be identified to support this suggestion. In addition, *T. alkalitolerans* JCM 18968^T, *T. lucentensis* QMT2^T and *T. xianhensis* P-4^T were also found to have the same sequence presented in different locations (Table 5.7.2). These miRNAs can have an influence on their expression and function at different genomic locations (Paczynska et al., 2015). It will be of great interest to identify the target mRNAs of these miRNAs, and investigate

their roles and the mechanisms of gene regulation in the physiology of these unique environmental bacteria.

Table 5.7.1 Conserved putative miRNAs in different species of bacteria of the genus *Thalassospira*.

miRNA sequence	***T. alkalitolerans* JCM 18968^T**	***T. australica* NP 3b2^T**	***T. lucentensis* QMT2^T**	***T. mesophila* JCM 18969^T**	***T. profundimaris* WP0211^T**	***T. tepidiphila* 1-1B^T**	***T. xianhensis* P-4^T**	***T. xiamenensis* M-5^T**
TTAATCCGGACCCATTAATTAT	T. alka_5p_4942		T. luce_5p_520914					
CATAATTAATGTGTTCGGAACT	T. alka_3p_4942		T. luce_3p_520914					
ATCAGGTCGAAGCCATGACCAT	T. alka_5p_1819		T. luce_5p_218949					
TCTGGCATCGGCGTTTCTATCG	T. alka_5p_4684		T. luce_5p_498538					
CGTCGTCTGATCCGCTTTGCCA	T. alka_3p_4684		T. luce_3p_498538					
AGCGACAACGCCGGTGGGATCA					T. profu_5p_37885	T. tepi_5p_24769		
TGCCACCGGCGTTGTTGTCTTC					T. profu_3p_37885	T. tepi_3p_24769		
AAGAAGCAGCGTCGGCCAGCCA					T. profu_5p_14495	T. tepi_5p_9431		
GCCGGCGCTGCCTCAACTCGTT					T. profu_3p_14495	T. tepi_3p_9431		
ATCAAAAAGGCGGAGCTGATTT					T. profu_5p_9602	T. tepi_5p_6592		
CTCCGCCTTTTTTTTGTTCGAG					T. profu_3p_9602	T. tepi_3p_6592		

miRNA sequence	*T. alkalitolerans* JCM 18968^T	*T. australica* NP 3b2^T	*T. lucentensis* QMT2^T	*T. mesophila* JCM 18969^T	*T. profundimaris* WP0211^T	*T. tepidiphila* 1-1B^T	*T. xianhensis* P-4^T	*T. xiamenensis* M-5^T
AATCATCGATCCGTTGATCTTC					T. profu_5p_49438	T. tepi_5p_33046		
AACTTTGTGCAGCTGTTTGGTC					T. profu_3p_49438	T. tepi_3p_33046		
CGCGGCGGTGGCGTTGCCGAAC					T. profu_5p_45214	T. tepi_5p_29936		
AACGTGATGGCGTCATGCACCG					T. profu_5p_45214	T. tepi_3p_29936		
CGGTTGCAATTGCGACCACCAC					T. profu_5p_29989	T. tepi_5p_19433		
CCTTATAGCGGAATGCGCCCTG					T. profu_3p_29989	T. tepi_3p_19433		
AACAAAACCCGCAAGGCCAATG					T. profu_5p_41148	T. tepi_5p_26936		
TTGCGGGTTTTGCTGTGATGTT					T. profu_3p_41148	T. tepi_3p_26936		
GGGGGGAAAAGTTCCCTTGCCG					T. profu_5p_54785	T. tepi_5p_36487		
TTGCCGAACGGCTGAAAGAGCT					T. profu_3p_54785	T. tepi_3p_36487		
CAATCTGTTGCAGTGCCTGATC					T. profu_5p_30109	T. tepi_5p_19555		
CTGATCTGCTTCGTTACGGATA					T. profu_3p_30109	T. tepi_3p_19555		
TGCCTATCGCGTCGACGAGGTG					T. profu_5p_7879	T. tepi_5p_5471		
TGTCGAGGCGGCTGGTCTGCGT					T. profu_3p_7879	T. tepi_3p_5471		

miRNA sequence	*T. alkalitolerans* JCM 18968^T	*T. australica* NP 3b2^T	*T. lucentensis* QMT2^T	*T. mesophila* JCM 18969^T	*T. profundimaris* WP0211^T	*T. tepidiphila* 1-1B^T	*T. xianhensis* P-4^T	*T. xiamenensis* M-5^T
CACCGATGTCGAAAGATCTTCG					T. profu_5p_778	T. tepi_5p_572		
TTCCATATCGTGTCGTTATTCA					T. profu_3p_778	T. tepi_3p_572		
AATTGTATGTGCAATAATGCGA					T. profu_5p_32260	T. tepi_5p_20938		
GCGTTCGGAGGATTGCACATGC					T. profu_3p_32260	T. tepi_3p_20938		
CTCATGAGTAATGTGTTCGGAA					T. profu_3p_3800	T. tepi_3p_2570		
TGTGATGGTTTCTTCTATCGCA					T. profu_5p_19048	T. tepi_5p_11782		
GTCGGTGGCGGTAACACCGCGG					T. profu_3p_19048	T. tepi_3p_11782		
TTTCAACAACGCCCGTTGATTG					T. profu_5p_38661	T. tepi_5p_25251		
ATTGAAATCCCCCGCCTAAACC					T. profu_3p_38661	T. tepi_3p_25251		
ATTTTGTACCTGATGAAACGGC					T. profu_5p_42425	T. tepi_5p_27927		
CGTTTTGTTAGGTGTTAACCTG					T. profu_3p_42425	T. tepi_3p_27927		
TATGCCAACAATCCGACCGGGT					T. profu_5p_24235	T. tepi_5p_15757		
GCGGTCTGGATGTTGGCCTGCC					T. profu_3p_24235	T. tepi_3p_15757		
AGCAAAAGCTGCCTAATTAAGG					T. profu_5p_21694	T. tepi_5p_13698		

miRNA sequence	*T. alkalitolerans* JCM 18968^T	*T. australica* NP 3b2^T	*T. lucentensis* QMT2^T	*T. mesophila* JCM 18969^T	*T. profundimaris* WP0211^T	*T. tepidiphila* 1-1B^T	*T. xianhensis* P-4^T	*T. xiamenensis* M-5^T
CTTCTGCTTTACAGACAGAATT					T. profu_3p_21694	T. tepi_3p_13698		
TGTCTTTTTCTGACGTTTTTTC					T. profu_5p_49945	T. tepi_5p_33271		
CGTTTTTTCTCAAAAAAGGGTT					T. profu_3p_49945	T. tepi_3p_33271		
TTGTCTGTCAAACAGGCAAGGA					T. profu_5p_17170	T. tepi_5p_11264		
AAGGATTGCGGTCGGCCTTACT					T. profu_3p_17170	T. tepi_3p_11264		
TGACGCAGAGGCTTTCTCTCAT					T. profu_5p_51572	T. tepi_5p_34376		
AGGTGGCCTTTGGATCACCCGG					T. profu_3p_51572	T. tepi_3p_34376		
GTCGGCGTTGTCGCGCTGTTCA					T. profu_5p_12216	T. tepi_5p_8405		
TTCAAGGAGCCGCTGCATGTTG					T. profu_3p_12216	T. tepi_3p_8405		
AGACGTGACCTTCGGGTCGCGT							T. xian_5p_16668	T. xiam_5p_26826
CGTCTTTTTTATTGTCTGGTGG							T. xian_3p_16668	T. xiam_3p_26826
CAATTAAAAACCCCCTCAGGCG							T. xian_5p_6822	T. xiam_5p_11702

miRNA sequence	*T. alkalitolerans* JCM 18968^T	*T. australica* NP 3b2^T	*T. lucentensis* QMT2^T	*T. mesophila* JCM 18969^T	*T. profundimaris* WP0211^T	*T. tepidiphila* 1-1B^T	*T. xianhensis* P-4^T	*T. xiamenensis* M-5^T
AGGGGTTTTTTAATTGGTAGCC							T. xian_3p_6822	T. xiam_3p_11702
CAATTTAAACATTGTCACCATG		T. aust_3p_49513				T. tepi_3p_22906		
ATAATGACGTCCGTTGCGAC			T.luce_5p_10500			T.tepi_5p_8152		
TCACAGTCGAGACGCTCTCTCACC			T.luce_3p_22524	T.meso_3p_3562				
GTCCGGTGGTCTGGGCACCATG					T.prof_5p_36012	T.tepi_5p_22622		
ATCCTGCCCCCGCAACCA					T.prof_5p_14768	T.tepi_5p_8570		

Table 5.7.2 miRNAs present in different locations of the same *Thalassospira* species.

miRNA sequence	***T. alkalitolerans* JCM 18968^T**		***T. lucentensis* QMT2^T**		***T. xianhensis* P-4^T**	
AATTAATGGGTCCTGACC	T.alka_3p_6323	T.alka_3p_6324				
ATTCCCGCGTGCGCGGGAATGA					T. xian_3p_2740	T. xian_3p_2738
TTCGGTGCTCACGTACTTTTAG			T. luce_5p_528656	T. luce_5p_528636		
TGCGCTCCGATGCGCGTGAACC			T. luce_3p_528656	T. luce_3p_528636		
TCGTGCGTCAGCTTGGCGTGAC			T. luce_5p_389406	T. luce_5p_389408		
TCACCCGACCTGACCATGGTCG			T. luce_3p_389406	T. luce_3p_389408		

5.8 Summary

The information presented in this chapter described an investigation of the miRNA population found in the bacteria of the genus *Thalassospira*, including *T. australica* NP 3b2^T, *T. lucentensis* QMT2^T, *T. profundimaris* WP0211^T, *T. xiamenensis* M-5^T, *T. alkalitolerans* JCM 18968^T, *T. mesophila* JCM 18969^T, *T. povalilytica* Zumi 95^T, *T. tepidiphila* 1-1B^T and *T. xianhensis* P-4^T. Over 242 million reads, with 18 to 33 nucleotides in length using high-throughput sequencing technology, were generated from nine bacterial species. Using miRDeep and CID-miRNA analyses, a total of 984 putative miRNAs were identified in 9 libraries generated from nine *Thalassospira* species with typical miRNA length of 19 - 25 nucleotides. Among the detected putative miRNAs, *T. lucentensis* QMT2^T was found to have highest number of miRNAs detected with 333 sequences, *T. mesophila* JCM 18969^T and *T. povalilytica* Zumi 95^T have found to have only 7 and 8 miRNA sequences, respectively. The remaining miRNA sequences belonged to *T. australica* NP 3b2^T (165 putative miRNAs), *T. profundimaris* WP0211^T (170 putative miRNAs), *T. xiamenensis* M-5^T (38 putative miRNAs), *T. alkalitolerans* JCM 18968^T (53 putative miRNAs), *T. tepidiphila* 1-1B^T (131 putative miRNAs) and *T. xianhensis* P-4^T (79 putative miRNAs). These putative miRNAs were not found to be shared in *E. coli* DH10B and *S. mutans ATCC* 25175. 57 conserved putative miRNAs were, however, found in different species of the genus *Thalassospira*, and 6 miRNA sequences were found to be present in the same species of *T. alkalitolerans* JCM 18968^T, *T. lucentensis* QMT2^T and *T. xianhensis* P-4^T at different locations. Here, the first comprehensive list of computationally identified putative miRNAs in 9 bacterial species of the genus *Thalassospira* was presented.

Chapter 6.

Identification of salt responsive microRNAs and their targets in barley genotypes varying in salt stress response using small RNA sequencing

6.1 Overview

The recent discovery of microRNAs (miRNAs) as post-transcriptional regulators has confirmed the role of these small RNAs in response to environmental stresses. miRNAs have been reported as being salt responsive in many plant species such as *Arabidopsis* (Liu et al., 2008), rice (Zhao et al., 2009) and wheat (Eren et al., 2015). miRNAs are endogenous, typically 19-25 nucleotides long, single-stranded, non-coding RNAs that regulate the expression of their target, mRNA, by the processes of cleavage or translation inhibition (Guleria, 2011). In summary, mature miRNAs in plants are produced, by their own genes, into primary-miRNAs (pri-miRNAs) and then precursor-miRNAs (pre-miRNAs) in the nucleus. The miRNA is then exported to the cytoplasm and incorporated into the RNA-induced silencing complex (RISC), guiding the argonaute (AGO) protein to cleave or inhibit mRNA translation (as discussed in Chapter 2). To date, over 5000 miRNAs from *Arabidopsis, Brachypodium*, rice, maize, and sorghum have been identified and deposited into the miRBase v21.0 (http://www.mirbase.org).

miRNAs have been extensively studied in plants (as discussed in Chapter 2); however, there has been little research as yet on miRNA responses to abiotic stress in barley, the most important cereal crops world-wide, ranking fourth amongst the cereal crops. So far, up to 100 miRNAs have been identified in leaf tissue through deep sequencing (Schreiber et al., 2011) and 126 conserved and 133 barley-specific miRNAs found in different tissues, e.g., roots, stems, leaves and spikes (Lv et al., 2012). Expression of barley miRNAs has also been investigated under several abiotic stress conditions such as drought (Kantar et al., 2010), phosphorus stress (Hackenberg et al., 2013b), boron stress (Ozhuner et al., 2013), and heat stress (Kruszka et al., 2014), resulting in 71 miRNAs being deposited into the miRBase. At the time of the study, only 44 miRNAs that respond to salt stress, one of the most significant stress factors, have been identified in the barley cultivar Morex (Deng et al., 2015). In this chapter, the specific expression of miRNA and potential mRNA targets in the salt sensitive or tolerant barley cultivars, Arivat and Calmariout respectively, were studied. Deep small RNA transcriptome sequence data were used to identify the miRNAs. Some of the miRNAs that were identified, as well as their potential mRNA targets, were further validated by cloning and sequencing, and/or quantitative PCR.

6.2 Identification of expressed miRNAs in barley leaf small RNA-seq transcriptome data

The next-generation small RNA-Seq method was employed for detection of miRNAs in barley plants (Arivat and Calmariout cultivars). Four libraries were prepared using the pooled total RNA extracted from the leaf tissue of two independent plants in four groups: (i) barley (cv. Calmariout) treated with 150 mM NaCl for 12 h, (ii) barley (cv. Arivat) treated with 150 mM NaCl for 12 h, (iii) barley (cv. Calmariout) grown in the absence of salt, and (iv) barley (cv. Arivat) grown in the absence of salt. Pooled RNA samples have been applied in other studies (Mizuno et al., 2010, Ando and Grumet, 2010) to reduce the likelihood of any biological variations between transcriptomes impacting on the data; this technique, however, does not allow any assessment of variability. Each library was then loaded onto an Illumina Genome Analyser IIx for sequencing. Over 60 million reads, with 18 to 28 nucleotides in length, were obtained from four libraries. According to the recent release of the sequenced barley genome (October 2012; The International Barley Genome Sequencing Consortium (IBSC), 2012), the small RNA sequence reads were aligned to the barley genome sequence (cv. Bowman) using Burrows-Wheeler Aligner (BWA) software (Li and Durbin, 2009). The results showed that up to 95% of these reads aligned with the barley genome sequence (cv. Bowman) in both the control and stressed libraries of both cultivars (Table 6.2.1). The unaligned sequences may belong to viral RNA in plants were infected during the growth stage, or mRNA fragment or other RNA sources arising from contamination through the process. The aligned reads were then subjected to further analysis for miRNA detection.

Table 6.2.1 Small RNA-Seq data yield from Genome Analyzer IIx sequencing.

	Arivat cultivar		**Calmariout cutivar**	
	Control sample	**Salt stress sample**	**Control sample**	**Salt stress sample**
Original read length (nt)[a]	18-35	18-35	18-35	18-35
Original number of reads after short sequence removal (million)[b]	15,408,211	19,232,458	15,043,963	15,578,765
Number of reads aligning to barley genome (million)[c]	14,502,017	17,996,421	13,966,038	14,676,274
% reads aligned	94.1	93.6	92.8	94.2

[a]: the reads obtained from this experiment was set from 18-35 nt using an illumina genome analyser with low error rate. [b]: total reads were obtained from the leaf tissue of salt stressed and control plants. [c]: reads were aligned to the barley genome sequence (cv. Bowman) using the BWA algorithm with the default settings.

In order to identify miRNA populations, the Mireap software (http://sourceforge.net/projects/mireap/) was employed to detect millions of short sequences generated from the dataset. The Mireap software has been recommended for predicting miRNAs (Li et al., 2012b) that widely occur in wheat (Ma et al., 2015), rice (Yi et al., 2013b), barley (Lv et al., 2012) and maize (Gu et al., 2013). After analysis, the program provided a list of miRNA genes based on some principal criteria: (i) miRNA* located in opposite stem-arms with two-nucleotide 3' overhangs; (ii) no more than 4 bases mismatched between the miRNA and miRNA* located in the other arm; (iii) asymmetric bulges, especially within miRNA-miRNA* duplex, of minimal size and frequency (usually less than one) (Meyers et al., 2008). The result included 231 miRNA precursor genes that satisfied the above criteria. 112 mature miRNAs were located in the 5' arm and 124 mature miRNAs were located in the 3' arm of the pre-miRNAs. Five complementary pre-miRNAs were identified within two mature sequences, located in both arms, and considered as miRNA and miRNA* sequences.

6.3 Identification of salt responsive miRNAs

Identification of the miRNAs that responded to salinity was based on the *p* value and the change in their expression abundance. Differential expression of miRNAs was calculated by dividing the adjusted read counts between libraries. The miRNAs with fold-change < -1 or > 1 in either Calmariout or Arivat cultivar, along with the adjusted *p* value < 0.05, were considered as downregulated or upregulated upon salt stress, respectively. Based on the criteria, 41 salt responsive miRNAs were identified with 20 to 24 nucleotides in length (Table 6.3.1). The length of these miRNAs was found to be consistent with the size distribution of the miRNAs previously reported (Liang et al., 2010, Ozhuner et al., 2013). These reads were then mapped to the barley miRNA sequences and other plant miRNA species deposited into miRbase v.21, resulting in the detection of three known barley miRNAs (hvu-MIR159a/b, hvu-MIR171 and hvu-MIR5048) along with other 11 orthologs with no more than 3 mismatches (Table 6.3.2). The remaining 25 candidates were categorised as putative novel barley miRNAs that had not been previously reported in barley miRNAs studies (Kruszka et al., 2013, Wang et al., 2013, Schreiber et al., 2011, Kantar et al., 2010, Deng et al., 2015, Hackenberg et al., 2013b, Ozhuner et al., 2013). The existence of these miRNAs in other barley varieties was also demonstrated by aligning these sequences to the recent release of two barley genome sequences, for the Morex and Barke cultivars. These results confirmed the presence of most of the miRNA candidates in these cultivars (Table 6.3.1), including the presence of five known barley miRNAs.

Table 6.3.1 Barley miRNAs identified by miReap.

miRNA	Location	Start-End position	Sequence (5'-3')	Number of reads used by miReap
Known miRNAs				
hvu-MIR171	bowman_contig_863591 morex_contig_1572026 barke_contig_271341	1761-1781 1168-1188 2227-2247	TGTTGGCTCGACTCACTCAGA	NC
hvu-MIR159a	bowman_contig_845099 morex_contig_132996 barke_contig_1783742	2389-2409 5815-5835 5813-5833	TTTGGATTGAAGGGAGCTCTG	NC
hvu-MIR159b	bowman_contig_845099 morex_contig_132996 barke_contig_1783742	2389-2409 5815-5835 5813-5833	TTTGGATTGAAGGGAGCTCTG	NC
hvu-MIR5048a	bowman_contig_14776 morex_contig_117145 barke_contig_1789351	1187-1208 3104-3125 1090-1111	TATTTGCAGGTTTTAGGTCTAA	NC
hvu-MIR5048b	bowman_contig_14776 morex_contig_117145 barke_contig_1789351	1187-1208 3104-3125 1090-1111	TATTTGCAGGTTTTAGGTCTAA	NC
Novel and homologous miRNA candidates				
SUT_hvu_mir_000173	bowman_contig_857361 morex_contig_55859 barke_contig	6879-6900 6879-6900 NA	AGACTAGGACGCCGCCGGAGAA	65

miRNA	Location	Start-End position	Sequence (5'-3')	Number of reads used by miReap
SUT_hvu_mir_000108	bowman_contig_42529 morex_contig barke_contig_1808600	165-186 NA 1250-1271	CACGAGGGCTCTGCTCGCTGAT	3
SUT_hvu_mir_000174	bowman_contig_857828 morex_contig_159434 barke_contig_2784321	1313-1335 2918-2940 970-992	TTGCATCTCTCGGGTCGTTCCAG	44
SUT_hvu_mir_000045	bowman_contig_196257 morex_contig_133519 barke_contig_118619	3202-3225 325-348 3121-3144	GCTTCTTGCTGATGGTGTTATTCC	166
SUT_hvu_mir_000163	bowman_contig_851924 morex_contig_1567590 barke_contig_1791675	3777-3800 3629-3652 221-244	CATATATGTAGTGCTGTAAGAAGA	57
SUT_hvu_mir_000150	bowman_contig_845316 morex_contig_137990 barke_contig_1781947	3009-3030 2681-2702 3003-3024	TTTGGATCGAAGGGAGTTTTTT	413
SUT_hvu_mir_000133	bowman_contig_73670 morex_contig_68759 barke_contig_300209	10386-10408 4130-4152 4101-4123	GAACGATTTGAGGCGATTTGAAC	59

miRNA	Location	Start-End position	Sequence (5'-3')	Number of reads used by miReap
SUT_hvu_mir_000075	bowman_contig_1997879 morex_contig_1563409 barke_contig_451354	48-71 2254-2277 2242-2265	AAGGAAACTGGGGCAGTGGCATAT	59
SUT_hvu_mir_000049	bowman_contig_1982219 morex_contig_1563116 barke_contig	8183-8204 2319-2340 NA	TTCTCCGTCGACGTCATCTTTG	7
SUT_hvu_mir_000050	bowman_contig_1982666 morex_contig_1577449 barke_contig_270170	1833-1854 2056-2077 2048-2069	AAACAGATCTCAAGGATCTATT	91
SUT_hvu_mir_000081	bowman_contig_2061088 morex_contig_295426 barke_contig	54-75 415-436 NA	TCTTCTGAAGCTGTGGAATGTC	1
SUT_hvu_mir_000127	bowman_contig_69168 morex_contig_135493 barke_contig_373400	1438-1459 2701-2722 779-800	CTTGCTCCCTTTCATTTTTTGT	356
SUT_hvu_mir_000029	bowman_contig_13160 morex_contig_8857 barke_contig	3847-3868 204-225 NA	CGCCGTCGCTTCGTCGTACATC	22

miRNA	Location	Start-End position	Sequence (5'-3')	Number of reads used by miReap
SUT_hvu_mir_000025	bowman_contig_129321 morex_contig_162791 barke_contig_300037	2064-2086 2193-2215 2479-2501	GTGCTTATTGACGGTCCAGTGCT	95
SUT_hvu_mir_000095	bowman_contig_267356 morex_contig_45828 barke_contig_278740	6160-6181 6105-6126 4380-4401	TTCTTCCCAGCAATGGGCATAT	148
SUT_hvu_mir_000026	bowman_contig_129321 morex_contig_162791 barke_contig_300037	2336-2358 2465-2487 2207-2229	GAGCTTATTGACGGTCCAGTGCT	28
SUT_hvu_mir_000158	bowman_contig_850044 morex_contig_48306 barke_contig_74785	4206-4229 297-320 296-319	ACATGCATCGTGCTGGGGAGAAAA	16
SUT_hvu_mir_000084	bowman_contig_21525 morex_contig_49772 barke_contig_240012	467-488 3175-3196 63-84	TGCTGCGTCGACGCCATCAGCC	27
SUT_hvu_mir_000112	bowman_contig_62402 morex_contig_51004 barke_contig	974-996 1059-1081 NA	CCTACTAACGCGTTTCCTTTCCA	11

miRNA	Location	Start-End position	Sequence (5'-3')	Number of reads used by miReap
SUT_hvu_mir_000027	bowman_contig_130167 morex_contig_2544689 barke_contig	1617-1640 113-136 NA	ATGGGATTGCTCGTATTATAGGTC	18
SUT_hvu_mir_000142	bowman_contig_836985 morex_contig barke_contig	88-108 NA NA	CTATGTAGACTTTTGTTTAAA	7
SUT_hvu_mir_000046	bowman_contig_1981154 morex_contig_135892 barke_contig_248721	5599-5620 5563-5584 44-65	GGAACGTTGGCTGGCTCGAGGC	5
SUT_hvu_mir_000023	bowman_contig_127644 morex_contig_135760 barke_contig_268557	2144-2165 5924-5945 2123-2144	CTGGGAGACTTCTAACTTAAAT	3
SUT_hvu_mir_000094	bowman_contig_259684 morex_contig_1571462	571-592 3345-3366	TCCATCCATCCGATCCCAGGAG	75
SUT_hvu_mir_000039	bowman_contig_1495473 morex_contig_187775 barke_contig_338329	616-639 13854-13877 1410-1433	TGAATTTGTTTAACTAGAATTTAT	65
SUT_hvu_mir_000034	bowman_contig_144352	1002-1023	AGCAAATGATGAGCTTACTCGG	14

miRNA	Location	Start-End position	Sequence (5'-3')	Number of reads used by miReap
	morex_contig_39118 barke_contig_272352	2074-2095 922-943		
SUT_hvu_mir_000228	bowman_contig_978416 morex_contig_242192 barke_contig	39-59 45-65 NA	TTTGCCATCAGCCTTGGGGCT	166
SUT_hvu_mir_000079	bowman_contig_2025952 morex_contig barke_contig	319-339 NA NA	AAGTTGGGCAATAATGTTGTA	7
SUT_hvu_mir_000180	bowman_contig_861862 morex_contig_158387 barke_contig_57973	9821-9844 5277-5300 5546-5569	CTTTCTGAACTCTTCTATTCCAGG	112
SUT_hvu_mir_000041	bowman_contig_159895 morex_contig barke_contig_212714	761-783 NA 181-203	TTGGCGGAGCTCCTGCCCTATTT	3
SUT_hvu_mir_000135	bowman_contig_75192 morex_contig_2542585 barke_contig_386781	18524-18545 165-186 1769-1790	ATTATGAAGACCCGATCGGATT	10
SUT_hvu_mir_000062	bowman_contig_1986407	144-163	GCGAACGAACGATCTAAACT	15

miRNA	Location	Start-End position	Sequence (5'-3')	Number of reads used by miReap
	morex_contig_135509 barke_contig	6437-6456 NA		
SUT_hvu_mir_000186	bowman_contig_866395 morex_contig_39847 barke_contig_56519	1988-2009 5518-5539 1849-1870	TGGCGCTCCTGCTGCGCTCTCC	36
SUT_hvu_mir_000061	bowman_contig_1986342 morex_contig_368569 barke_contig_512712	397-416 4359-4378 3693-3712	TAGGTTCATCCGTTGTCGCT	26
SUT_hvu_mir_000043	bowman_contig_1663310 morex_contig barke_contig	57-77 NA NA	GGCGGATGTAGCCAAGTTGAG	541
SUT_hvu_mir_000010	bowman_contig_11073 morex_contig_39705 barke_contig_2786991	235-254 16450-16469 183-202	TTTGATTAATCCGGTCTCGA	4

"NC": not counted

"NA": not available

Table 6.3.2 Barley miRNA orthologues in other organisms.

Barley miRNA	**Orthologous miRNA**	**Organism**	**Reported target for this miRNA**
SUT_hvu_mir_000010	ppt-miR1067	*Physcomitrella patens*	Unknown
SUT_hvu_mir_000039	csi-MIR3949	*Citrus sinensis*	Unknown
SUT_hvu_mir_000045	ath-MIR5658	*Arabidopsis thaliana*	Unknown
SUT_hvu_mir_000046	osa-miR166h-5p	*Oryza sativa*	HD-Zip transcription factors (Jones-Rhoades and Bartel, 2004)
SUT_hvu_mir_000049	osa-miR5075	*Oryza sativa*	Unknown
SUT_hvu_mir_000061	mtr-MIR2592al	*Medicago truncatula*	Unknown
SUT_hvu_mir_000108	sbi-miR171b	*Sorghum bicolor*	Unknown
SUT_hvu_mir_000127	osa-miR1879/2863b/5535	*Oryza sativa*	Unknown
SUT_hvu_mir_000150	ath-miR159b-3p aly-miR159b-3p	*Arabidopsis thaliana* *Arabidopsis lyrata*	MYB transcription factor (Dryanova et al, 2008)
SUT_hvu_mir_000180	bdi-miR5185f	*Brachypodium distachyon*	Unknown
SUT_hvu_mir_000228	aly-MIR4231	*Arabidopsis lyrata*	Unknown

As seen from the data presented in Table 6.3.1, only 41 out of 231 expressed miRNAs were able to be categorised as salt responsive miRNAs. The other 190 low-expressed miRNAs may play a role in other environmental stresses or be expressed to a greater extent under different conditions. Detection of hvu-MIR159, hvu-MIR171 and hvu-MIR5048 as barley salt responsive miRNAs is in agreement with Deng and his colleagues (2015), who reported both hvu-MIR171 and hvu-MIR5048 as being salt responsive miRNAs in the Morex barley cultivar. In addition, homologues of miRNAs hvu-MIR171 and hvu-MiR159 have been previously reported as being salt responsive miRNAs in *Arabidopsis* (Liu et al., 2008) and wheat (Lu et al., 2011). Previous studies

also showed the ability of these miRNAs to respond to other abiotic stresses such as drought stress (Kantar et al., 2010) or boron stress (Ozhuner et al., 2013). These findings supported the role of these conserve miRNAs being in response to salt stress as well as other abiotic stress. So far, Deng et al. (2015) have reported five novel miRNAs as being salt responsive genes in barley. The present study contributed 25 miRNAs as new salt responsive miRNAs expressed in barley leaves. This number is rather limited, possibly due to the fact that only leaf RNA extraction was performed, together with omission of the miRNAs that were not expressed in concentrations above the detection threshold (10 reads per sample on average). Most sequences of the 41 salt-responsive miRNAs could be identified in the three sequenced barley genomes of the Bowman, Morex and Barke cultivars, while some sequences were found only in one of these cultivars, possibly due to species-specific miRNA expression taking place, or differences in gene coverage (Morex 82%, Bowman 71%, Barke 26%) (Goff et al., 2014).

6.4 Expression profile of miRNAs in barley genotypes responding to salt stress

Based on the normalized read counts from high-throughput sequencing, expression of miRNA in the Calmariout cultivar was generally higher than obtained for the Arivat variety. Some conserved miRNAs (hvu-MIR159 and hvu-MIR5048) and novel miRNA (SUT_hvu_mir_000186) were observed to be expressed abundantly in each library of both cultivars, while hvu-MIR171 displayed the lowest expression, with only around ten reads in each library. Among the conserved miRNAs, the abundant expression of hvu-MIR159 found in the present study was not in agreement with the study reported by Deng et al. (2015), who did not detect hvu-MIR159 in a Morex cultivar under salt stress conditions. The sequence of hvu-MIR159 was also found to be present in the Morex cultivar genome. These inconsistent findings could be due to tissue-specific expression, differences in salt concentration or the time of treatment compared to the current study (the RNA was extracted from leaf tissue under 150 mM NaCl for 12 h) and the study by Deng et al. (where the RNA was extracted from whole plant under salt stress conditions of 100 mM NaCl for 3 h, 8 h and 27 h). Noticeably, SUT_hvu_mir_000173 and SUT_hvu_mir_000158 were detected only in the control Calmariout cultivar while the SUT_hvu_mir_000010 was only presented in the Arivat cultivar (Table 6.4.1).

Table 6.4.1 Differential expression of miRNAs under salt stress conditions in barley (cv. Arivat and Calmariout) leaf tissue.

miRNA name	Calmariot control	Calmariot stress	Fold change	Arivat control	Arivat stress	Fold change
Known miRNAs						
hvu-MIR159a	339	190	↓1.78	204	144	↓1.42
hvu-MIR159b	1356	1126	↓1.20	1203	1255	↑1.04
hvu-MIR171	9	6	↓1.50	11	13	↑1.18
hvu-MIR5048a	402	292	↓1.38	263	270	↑1.03
hvu-MIR5048b	402	292	↓1.37	263	270	↑1.03
Homologous miRNAs						
SUT_hvu_mir_000010	**	**	**	27	13	↓2.08
SUT_hvu_mir_000039	10	12	↑1.20	11	6	↓1.83
SUT_hvu_mir_000045	4	7	↑1.75	22	49	↑2.23
SUT_hvu_mir_000046	143	87	↓1.64	100	119	↑1.19
SUT_hvu_mir_000049	23	14	↓1.64	14	10	↓1.40
SUT_hvu_mir_000061	13	10	↓1.30	12	15	↑1.25
SUT_hvu_mir_000108	46	88	↑1.91	36	62	↑1.72
SUT_hvu_mir_000127	103	110	↑1.07	1	2	↑2.00
SUT_hvu_mir_000150	117	82	↓1.43	111	67	↓1.66
SUT_hvu_mir_000180	7	7	*	23	19	↓1.21
SUT_hvu_mir_000228	48	44	↓1.09	31	33	↑1.06
Novel miRNA candidates						
SUT_hvu_mir_000023	40	34	↓1.18	33	27	↓1.22
SUT_hvu_mir_000025	28	35	↑1.25	35	39	↑1.11
SUT_hvu_mir_000026	105	94	↓1.12	86	151	↑1.76
SUT_hvu_mir_000027	19	24	↑1.26	30	31	↑1.03
SUT_hvu_mir_000029	249	176	↓1.41	174	167	↓1.04
SUT_hvu_mir_000034	20	16	↓1.25	18	19	↑1.05
SUT_hvu_mir_000041	15	16	↑1.06	15	15	*
SUT_hvu_mir_000043	75	134	↑1.78	150	67	↓2.24
SUT_hvu_mir_000050	42	53	↑1.26	46	65	↑1.41
SUT_hvu_mir_000062	15	12	↓1.25	16	23	↑1.44
SUT_hvu_mir_000075	19	14	↓1.35	17	11	↓1.55

miRNA name	Calmariot control	Calmariot stress	Fold change	Arivat control	Arivat stress	Fold change
SUT_hvu_mir_000079	25	15	↓1.67	26	31	↑1.19
SUT_hvu_mir_000081	10	17	↑1.70	15	18	↑1.20
SUT_hvu_mir_000084	256	167	↓1.53	155	174	↑1.12
SUT_hvu_mir_000094	15	15	*	19	24	↑1.26
SUT_hvu_mir_000095	20	23	↑1.15	13	18	↑1.38
SUT_hvu_mir_000112	14	9	↓1.56	12	12	*
SUT_hvu_mir_000133	6	11	↑1.83	12	17	↑1.42
SUT_hvu_mir_000135	11	16	↑1.45	11	5	↓2.20
SUT_hvu_mir_000142	116	202	↑1.74	183	137	↓1.33
SUT_hvu_mir_000163	162	274	↑1.69	396	837	↑2.11
SUT_hvu_mir_000158	83	**	**	**	**	**
SUT_hvu_mir_000173	41	**	**	**	**	**
SUT_hvu_mir_000174	21	11	↓1.91	21	14	↓1.50
SUT_hvu_mir_000186	1550	1248	↓1.24	1179	1421	↑1.20

↑ indicates up-regulation, ↓ indicates down-regulation, * indicates unchanged; ** indicates "not detected" in miRNA expression. A change of ≥ 1.5 fold indicates significant change under salt stress conditions.

The differential expression of salinity responsive miRNAs was calculated by comparing the expression profile of miRNAs obtained from the treated and untreated groups in each variety (cv. Arivat and Calmariout) based on normalized read counts from high-throughput sequencing. Most of the detected miRNAs were found to be down-regulated in the Calmariout cultivar and up-regulated in the Arivat cultivar subjected to salt stress. These miRNAs did not show any notable changes in their expression, varying from 1 to 2.2-fold changes. Out of the 41 detected miRNAs, ten showed up-regulation and seven displayed down-regulation in both barley lines. Both Calmariout and Arivat cultivars shared two miRNAs that were unchanged in their expressions under salt stress conditions. The remaining miRNAs showed an opposite expression, i.e., up-regulation in Calmariout and down-regulation in Arivat cultivar, or vice versa (Figure 6.4.1**)**. The difference in expression of miRNAs could be a result of the contrasting salt-resistance existing between the two cultivars. In addition, hvu-MIR171, hvu-MIR159 and hvu-MIR5048 were down-regulated in their expression in the Calmariout cultivar, but almost

unchanged in the Arivat cultivar after exposure to salinity conditions, confirming the results reported by Yin et al. (2012) on the miRNA expression in salt-tolerant and salt-sensitive cotton cultivars.

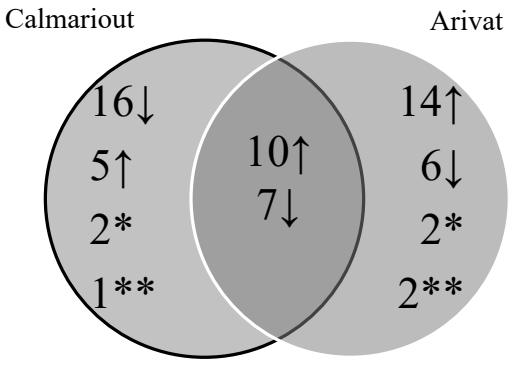

Figure 6.4.1 Venn diagram illustrating both common and unique differential miRNAs expression under salt stress conditions. ↑ indicates up-regulation, ↓ indicates down-regulation, * indicates an unchanged regulation and ** indicates when miRNA expressions were not detected.

6.5 Validation of miRNA expression

In order to validate the putative miRNA candidates from the small RNA-seq data, stem-loop RT-PCR and PCR-based directed cloning and sequencing were employed for testing the expression and exact sequence of amplified miRNAs. The barley (cv. Arivat and Calmariout) plants were grown under the same conditions as those samples used for small RNA-seq (as described in Chapter 3). Three independent plants were subjected to salinity conditions (150 mM NaCl for 12 h) as was done for the small RNA-seq trials, while the other three cultivars were grown under normal conditions for 14 days. The total RNA from the leaf tissue of both the control and salt stressed plants was used for testing the miRNA expression based on the stem-loop RT-PCR method described by Varkonyi-Gasic et al. (2007) (as detailed in Section 2.18.2). All of the 41 miRNA candidates were successfully amplified, showing a band between 50-75 bp in both cultivars (Figure 6.5.1).

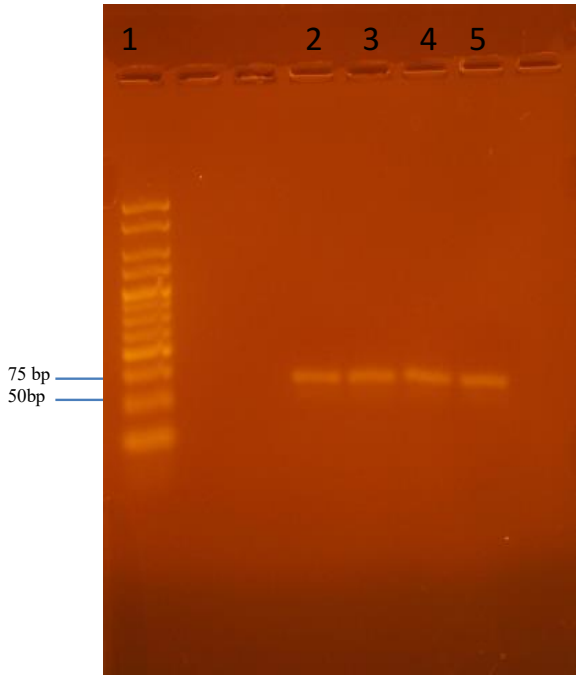

Figure 6.5.1 Example of a gel image showing successful amplification of hvu-miR159

Lane 1: HyperLadder^{TM} 25bp (Bioline), Lanes 2-5: PCR products from different RNA preparations

After showing successful amplification, some of the putative miRNA candidates (miR171, miR159, miR5048, SUT_hvu_mir_000026, SUT_hvu_mir_000029 and SUT_hvu_mir_000084) were selected randomly to enable to determination of the exact sequence of amplified miRNAs using PCR-based directed cloning and sequencing (as described in Chapter 3). The gel-purified miRNA candidates were cloned into pGEM®-T Easy. The presence of inserts was confirmed by the amplification of plasmid DNA, showing a band between 250-300 bp (the insertion of miRNAs into 178 nucleotide cloning sequence) (Figure 6.5.2). It was shown that the amplified sequences of conserved miRNAs (miR171, miR159 and miR5048) were consistent with the predicted sequences in both cultivars (Figure 6.5.3).

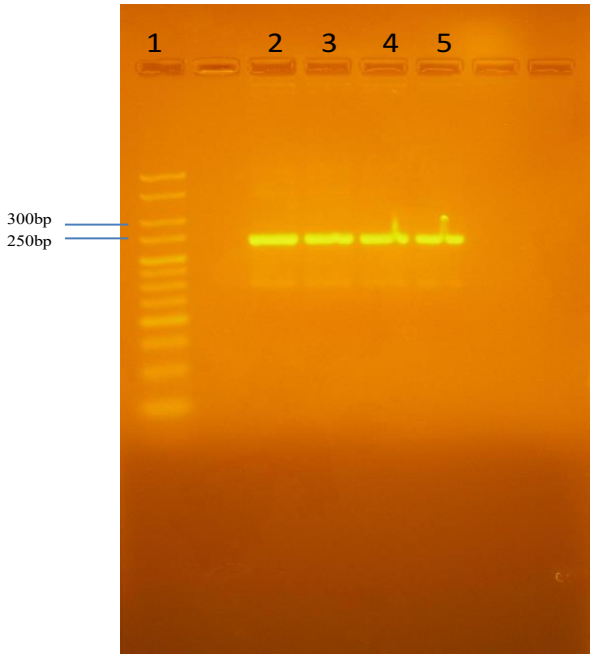

Figure 6.5.2 Example of a gel image showing successful insertion of hvu-miR159.

Lane 1: HyperLadder^{TM} 25bp (Bioline), Lanes 2-5: Plasmid PCR using vector-based primers (SP6 and T7) of clones.

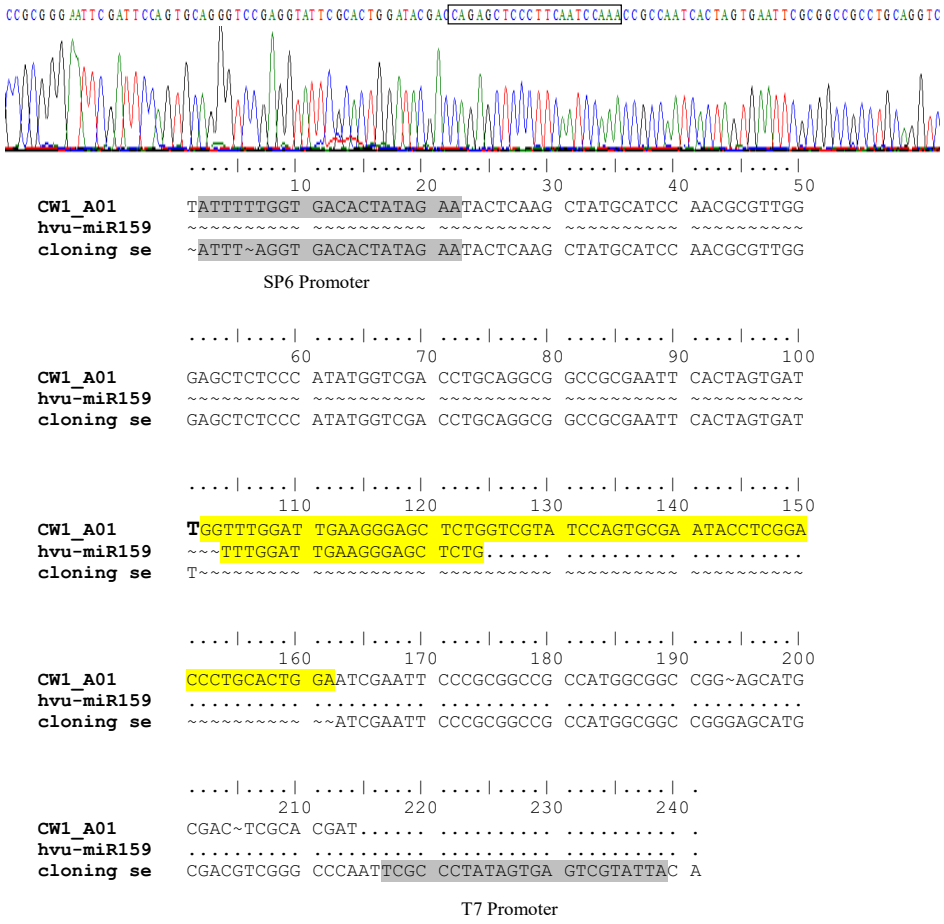

Figure 6.5.3 Cloning and validation of expression of hvu-MIR159 in both Calmariout and Arivat cultivars. The vector-based primers SP6 and T7 are highlighted in grey. The matching sequence located in the insert is highlighted in yellow**.**

6.6 Bioinformatics prediction of miRNA targets in barley

In order to understand the biological function of the detected salt responsive miRNAs, it is essential to identify the targets of these miRNAs. Hence, possible targets of the putative miRNAs were predicted using the psRNA Target Server under the default settings. The server was developed to enable the prediction of miRNA targets in plant according to two important analysis factors: (i) matching of the reverse complement between miRNA and target transcripts and (ii) evaluation of the target-site accessibility (Wassarman et al., 2001). The psRNA Target Server has been widely used for searching target genes in various plant species. In this study, the analytic quality of the psRNA Target Server was checked by a random selection of some miRNA sequences, predicting their targets using the psRNA Target Server, then compared to target validation data

reported in previous studies (Table 6.6.1). The well-validated target transcripts obtained experimentally warranted the quality of the server.

By using the psRNA Target Server, 68 targeted genes (transcripts) were found to be miRNA targets. Most of the targets were found in the HVGI database (Release 12). As shown in Table 6.6.2, some of the miRNAs had many predicted targets, whereas some did not have any specific target indicated in the database. Noticeably, the target of hvu-MIR171 could not be predicted in this study, whereas the scarecrow-like (SCL) transcription factor was reported as being its target in a previously reported study (Curaba et al., 2013). This discrepancy may be due to different severs being used for target prediction or the incomplete coverage of the barley genome in the HVGI database. Many of the predicted miRNA targets had known functions, whereas some could not be annotated. Most of the miRNAs were predicted to regulate their targets by cleavage, whereas a few did so by translational inhibition. Some of known functional miRNA targets have been reported in other plant species; these targets include the MYB transcription factor, serine/threonine-protein kinase and ubiquitin-conjugating enzyme E2. The MYB transcription factor and ubiquitin-conjugating enzyme E2 have role in plant responses to abiotic stress (detailed in section 2.11), whereas serine/threonine-protein kinase functions in the regulation of metabolic activity, plant growth and development (Parthibane et al., 2012) and the plant response to salt stress (Colaiacovo et al., 2010). In addition, some novel predicted targets were also found to have important roles in plant growth and in their response to stress, such as the blue copper binding protein, ABA-induced protein involving plant growth, development and adaptation to abiotic stress (Danquah et al., 2014), mitogen-activated protein kinase (MAPK) and indole-3-glycerol phosphate synthase functioning in signal transduction (Abass and Morris, 2013) and amino-acid biosynthesis (Colaiacovo et al., 2010). These targets may all have important functions in the development or physiology of the stress response of plants.

Table 6.6.1 Experimental validation of some miRNA targets predicted by psRNA Target Server (http://plantgrn.noble.org/psRNATarget/)

miRNA	Sequence	Predicted target	Experimental validation	Genomic library	References
hvu-miR156	UGACAGAAGAGAGUGAGCACA	Squamosa promoter-binding-like protein	qPCR	Barley	(Kantar et al., 2010)
hvu-miR159	UUUGGAUUGAAGGGAGCUCUG	Transcription factor GAMyb	qPCR	Barley	(Ozhuner et al., 2013)
miR164	UGGAGAAGCAGGGCACUUGCU	NAC transcription factor	qPCR	Barley	(Ozhuner et al., 2013)
hvu-miR5048	UAUUUGCAGGUUUUAGGUCUAA	Serine/threonine kinase-like protein	qPCR	Barley	(Ozhuner et al., 2013)
tae-miR408	CUGCACUGCCUCUUCCCUGGC	plantacyanin	qPCR	*Arabidopsis thaliana*	(Feng et al., 2013)
ppe-miR156a	UGACAGAAGAAAGAGAGCAC	Squamosa promoter-binding-like protein (SPL2)	qPCR	*Arabidopsis thaliana*	(Zhang et al., 2014b)
ppe-miR165a	UCGGACCAGGCUUCAUCCCCC	Homeobox-leucine zipper family protein	qPCR	*Arabidopsis thaliana*	(Zhang et al., 2014b)
ppe-mi171a	UGAUUGAGCCGUGCCAAUAUC	SLC6	qPCR	*Arabidopsis thaliana*	(Zhang et al., 2014b)
ppe-miR172a	AGAAUCUUGAUGAUGCUGC A	AP2 (APETALA 2) transcription factor	qPCR	*Arabidopsis thaliana*	(Zhang et al., 2014b)
ppe-miR393a	UCCAAAGGGAUCGCAUUGACC	AFB2 (auxin signaling F-box 2)	qPCR	*Arabidopsis thaliana*	(Zhang et al., 2014b)
ppe-miR394a	UUGGCAUUCUGUCCACCUCC	F-box protein	qPCR	*Arabidopsis thaliana*	(Zhang et al., 2014b)
osa-miR319b	UUGGACUGAAGGGUGCUCCC	PCF6	qPCR	Rice	(Thiebaut et al., 2012)

miRNA	Sequence	Predicted target	Experimental validation	Genomic library	References
ath-MIR169	CAGCCAAGGAUGACUUGCCGA	NF-YA	qPCR	*Arabidopsis thaliana*	(Sorin et al., 2014)
ctr-mir160	UGCCUGGCUCCCUGUAUGCCA	ARF	qPCR	*Arabidopsis thaliana*	(Shangguan et al., 2014)
miR395	CUGAAGUGUUUGGGGGAACUC	APS4; AST68; APS3	qPCR and 5'RACE	*Arabidopsis thaliana*	(Jagadeeswaran et al., 2014)
miR157	UUGACAGAAGAUAGAGAGCAC	SPL2	qPCR	*Arabidopsis thaliana*	(Wang et al., 2013)
miR162	UCGAUAAACCUCUGCAUCCAG	DCL1	qPCR	*Arabidopsis thaliana*	(Wang et al., 2013)
miR396	UUCCACAGCUUUCUUGAACUG	GRF3/4	qPCR	*Arabidopsis thaliana*	(Wang et al., 2013)
miR399	UGCCAAAGGAGAUUUGCCCUG	PHO2	qPCR	*Arabidopsis thaliana*	(Wang et al., 2013)
aly-miR846	UUGAAUUGAAGUGCUUGAAUU	Jacalin lectin protein	5'RACE	*Arabidopsis thaliana*	(Jia and Rock, 2013)
mir828	UCUUGCUUAAAUGAGUAUUCCA	myb	5'RACE	*Arabidopsis thaliana*	(Lin et al., 2012)

Table 6.6.2 Barley miRNA targets identified by psRNATarget (http://plantgrn.noble.org/psRNATarget/)

miRNA	Target gene name	Target Accession	Inhibition
hvu-MIR159a/b	Myb transcription factor	TC238438	Cleavage
	Triosephophate isomerase	TC252032	Translation
hvu-MIR171	Not predicted		
hvu-MIR5048a/b	Resistance protein	TC238421	Cleavage
	Serine/threonine kinase-like protein	TC238427	Cleavage
SUT_hvu_mir_000173	Alpha-amylase/trypsin inhibitor CMb precursor	BI947281	Cleavage
SUT_hvu_mir_000108	Histone H4	TC253420	Cleavage
	Ribosomal protein	AV928184	Cleavage
	GAMYB-binding protein	BE421464	Translation
SUT_hvu_mir_000174	Endoplasmin homolog precursor	BI953810	Cleavage
	ABA-induced protein	BG300592	Cleavage
SUT_hvu_mir_000045	Ubiquitin-conjugating enzyme E2	TC278879	Cleavage
	ABC transpoter-like protein	BU985949	Cleavage
	Translation initiation factor	TC266207	Cleavage
	Receptor-like protein kinase-like protein	TC277437	Cleavage
SUT_hvu_mir_000163	Blue copper binding protein	BE422246	Cleavage
SUT_hvu_mir_000150	Acyl0CoA thioester hydrolase-like	TC262911	Cleavage
	Phosphate/sulphate permeases	TC245082	Cleavage

miRNA	Target gene name	Target Accession	Inhibition
	HMG-CoA synthase	TC270221	Cleavage
SUT_hvu_mir_000133	Myotubularin-like protein	TC280553	Translation
SUT_hvu_mir_000075	Wiskott-Aldrich syndrome protein interacting protein	BI947640	Cleavage
SUT_hvu_mir_000049	Os04g0605900 protein	TC240485	Cleavage
	Ribosomal protein	BF259868	
SUT_hvu_mir_000050	Os02g0722700	TC248820	Translation
SUT_hvu_mir_000081	Mannosyl-oligosaccharide 1,2 alpha-mannosidase	TC269632	Cleavage
	ORF124	TC276717	Cleavage
	Retrotransposon protein	TC251317	Cleavage
SUT_hvu_mir_000127	Os04g0563000 protein	TC241010	Translation
	Triosephophate isomerase	BF267379	Cleavage
	Hydroxyproline-rich glycoprotein DZ-HRGP precursor	BG344335	Cleavage
	Beta-1,3-glucanase-like protein	GH212770	Cleavage
SUT_hvu_mir_000029	No predicted		
SUT_hvu_mir_000025	No predicted		
SUT_hvu_mir_000095	AGAP001055	TC252417	Translation
	Probable serine/threonine-protein kinase NAK	TC244889	Cleavage
	OJ000223_09.13 protein	TC245697	Cleavage
	Os04g0448200 protein	TC239896	Cleavage
SUT_hvu_mir_000026	No predicted		

miRNA	Target gene name	Target Accession	Inhibition
SUT_hvu_mir_000158	TAK14	CK566928	Translation
	Os08g0119300	TC270846	Cleavage
SUT_hvu_mir_000084	Fasciclin-like protein FLA13	TC281050	Cleavage
	Fasciclin-like protein FLA12	TC262725	Cleavage
	Os04g0205200	TC258343	Translation
SUT_hvu_mir_000112	No predicted		
SUT_hvu_mir_000027	Mitogen-activated protein kinase 6	BJ458579	Cleavage
	Indole-3-glycerol phosphate synthase, chloroplast precursor	TC251717	Cleavage
SUT_hvu_mir_000142	No predicted		
SUT_hvu_mir_000046	Os07g0301500	EX598729	Cleavage
	Predicted protein	TC270498	Cleavage
SUT_hvu_mir_000023	Chromosome chr11 scaffold_13	TC266762	Cleavage
SUT_hvu_mir_000094	Zinc finger CCCH domain-containing protein ZFN-like 2	TC243111	Cleavage
SUT_hvu_mir_000039	YeeE/YedE family protein	TC242650	Cleavage
SUT_hvu_mir_000034	Os07g0110400 protein	TC253713	Cleavage
	Predicted protein	TC263098	Cleavage
SUT_hvu_mir_000228	Alanyl-tRNA synthetase, mitochondrial precursor	TC259033	Cleavage
	Chromosome chr14 scaffold_27	TC256069	Cleavage
	S-adenosylmethionine decarboxylase	TC264523	Cleavage

miRNA	Target gene name	Target Accession	Inhibition
	Endoplasmin homolog precursor	TC238596	Translation
SUT_hvu_mir_000079	Metal-dependent membrane protease-like	TC253263	Cleavage
	PHD-finger family protein, expressed	GH224099	Cleavage
	Tyrosine phosphatase-like	TC263445	Cleavage
	Expressed protein	TC266098	Cleavage
SUT_hvu_mir_000180	No predicted		
SUT_hvu_mir_000041	No predicted		
SUT_hvu_mir_000135	Serpin	TC247643	Cleavage
SUT_hvu_mir_000062	ORF107c	TC261242	Cleavage
	60S ribosomal protein L5	TC258666	Cleavage
	Shikimate kinase	TC251208	Cleavage
	Cell wall-associated hydrolase	TC263920	Cleavage
SUT_hvu_mir_000186	No predicted		
SUT_hvu_mir_000061	Proteasome subunit alpha type	TC256259	Cleavage
	HAP3 transcriptional-activator	TC253639	Cleavage
SUT_hvu_mir_000043	Chloroplast 50S ribosomal protein L2	TC245676	Cleavage
	Os01g0770100	TC259394	Cleavage
	Papain family cysteine protease containing protein	BM817299	Translation
	Os08g130900	TC249520	Translation
SUT_hvu_mir_000010	Alternative oxidase	TC272517	Translation

6.7 Analysis of the correlated expression of selected miRNAs and their predicted targets using qRT-PCR

In order to analyse the correlation between the expression of miRNAs and their targets, three known miRNAs and their targets were selected for further investigation using qRT-PCR as they had been reported as being miRNAs responsive to salinity and other abiotic stress conditions (Deng et al., 2015, Ozhuner et al., 2013). The results showed that hvu-MIRNA159 and hvu-MIR5048 were down-regulated while hvu-MIR171 exhibited up-regulation in both cultivars. The expression showed a consistent trend to the miRNA profiles, with the exception that some discrepancy was present in the hvu-MIR171 expression in the Calmariout cultivar, possibly due to the limited expression of hvu-MIR171, which was detected at levels of less than 20 in the Calmariout cultivar. Furthermore, the expression pattern of up-regulation of hvu-MIR171 and down-regulation of hvu-MIR5048 under salt stress conditions, is in agreement with the data reported by Deng et al. (2015), validating the role played by these miRNAs in the response of barley to saline growth conditions.

The expression of the predicted and reported target of miR5048, a serine/threonine kinase-like protein (Ozhuner et al., 2013) was not detected in this study. Expressions of the predicted and reported target of hvu-MIR159, i.e., the MYB transcription factors (Kantar et al. 2010) and the reported target of hvu-MIR171, i.e., the scarecrow-like (SCL) transcription factor (Curaba et al., 2013), were found to be down-regulated in the Calmariout cultivar, but up-regulated in the Arivat cultivar (Figure 6.7.1). An inverse correlation between the hvu-MIR159 and its target was only observed in Arivat, and the hvu-MIR171 and its target in Calmariout. This correlation is not always observed in expression of miRNAs and their targets, as the targets are not regulated only by miRNAs. Additionally, a significant decrease in the mRNA targets in Calmariout could be a major factor contributing to this specific type of barley response to salt stress. The target of hvu-MIR159 was the MYB families of transcription factors (Kantar et al., 2010) that have diverse functions in plant developmental and metabolic processes, cell fate or abiotic and biotic stresses (Dubos et al., 2010). The scarecrow-like (SCL) transcription factor is the target of hvu-MIR171, affecting the degree of shoot development and timing of flowering. This factor has been reported to activate miR156 regulation. Hence, these

miRNAs may regulate the transcription of downstream genes that adjust the normal developmental processes, helping plants gradually adapt to salinity conditions.

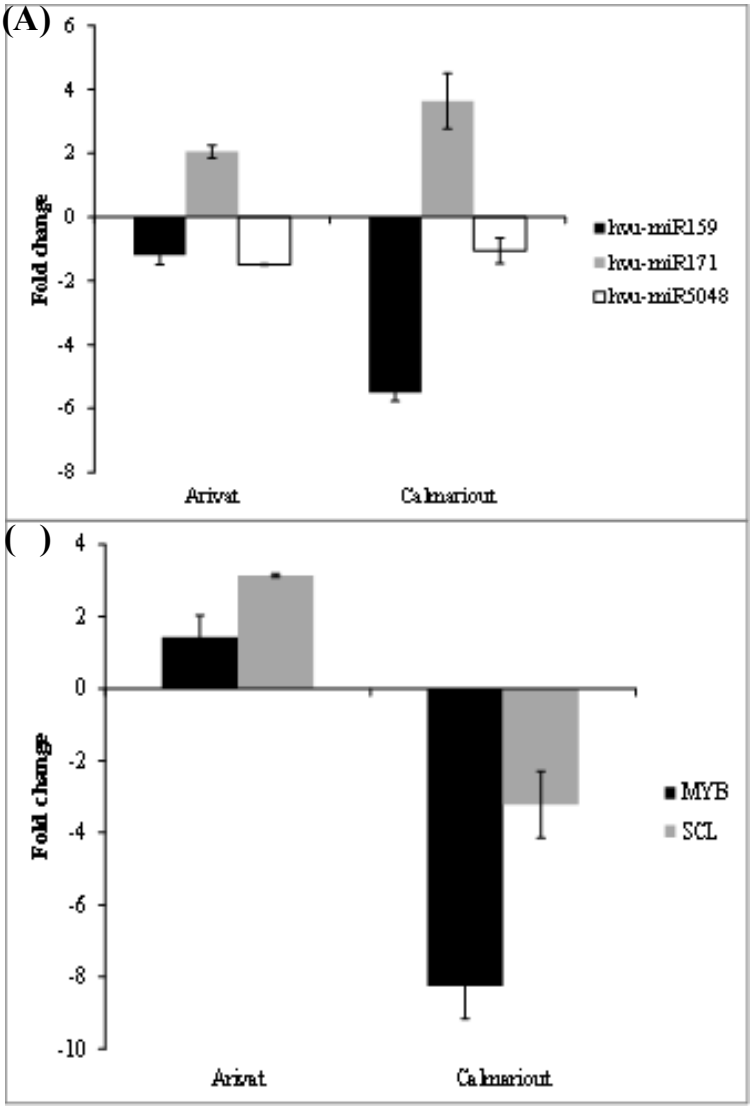

Figure 6.7.1 Expression level of selected miRNAs **(A)** and their targets **(B)** in the leaf tissue of two barley cultivars treated with 150 mM NaCl for 12 h. The error bars represent the standard deviation of triplicate measurements.

6.8 Summary

This chapter presented an investigation of the population of miRNAs in two distinct barley genotypes, the Arivat and Calmariout cultivars. Over 60 million reads were generated from barley leaves obtained from both cultivars, with 18 to 28 nucleotides in length using high-throughput sequencing technology. Using Mireap software, a total of 231 miRNAs were identified with typical miRNA lengths of 20-24 nucleotides. Among the detected miRNAs, 41 sequences were found to be salt responsive, including 5 known, 11 orthologs and 25 novel candidates. The miRNA expression pattern appeared to differ in the two barley genotypes, these being Arivat and Calmariout, which are salt sensitive and tolerant, respectively. Furthermore, 68 target genes were predicted using psRNA Target Server (http://plantgrn.noble.org/psRNATarget/). Most target transcripts were reported to be regulated by cleavage. Some targets have been reported to play an important role in plant responses to environmental changes and growth processes. In the present study, hvu-MIR171 and hvu-MIR5048 were confirmed as being salt responsive miRNAs as they displayed an expression that was consistent with the previous study. Regulation of these miRNAs on their target expression was supposed to attenuate plant growth to enable the plant to gradually cope with the stress growth conditions. The results could provide a better understanding of the miRNA expression profiles and their targets in two salt tolerant genotypes, allowing the tools of genetic selection and/or breeding to be further applied for salt tolerance.

Chapter 7.

Evaluation of salt tolerant and salt sensitive barley varieties using a physiological approach

7.1 Overview

As noted in extant literature (Chapter 2), salinity is a global issue that adversely influences the growth and development of plants, resulting in a reduction in productivity. High concentrations of salt in soil prevents the roots of plants from effectively taking up the water, and as such, is detrimental to plant growth (Munns and Tester, 2008). Barley (*Hordeum vulgare L.*) is one of the most important worldwide, ranking fourth amongst cereal crops based on production and is the second highest cultivated crop in Australia after wheat. Barley is used extensively in a number of food and beverage products in addition to being used extensively as an animal feed source. Although barley is a reasonably salt-tolerant crop (Flowers et al., 1977), its growth and yield is still adversely affected by high salt levels. Modern barley cultivars, to date, have become sensitive to abiotic and biotic stress due to the loss of genetic diversity during domestication compared to their landraces and wild ancestors (Russell et al., 2004). Thus, assessment of salt tolerance level of modern barley cultivars could offers immense potential for crop improvement via varietal selection or salt tolerance related gene studies.

Many criteria have been suggested for salt tolerance screening, e.g., growth rate (Greenway, 1962), plant survival rates under high salt levels (Sayed, 1985), germination rate (Von Well and Fossey, 1998), leaf and root elongation rate (Cramer and Quarrie, 2002), leaf injury and reduction of CO_2 assimilation (James et al., 2002) or K^+/Na^+ discrimination (Asch et al., 2000). These criteria are not usually related to each other, and therefore result in different salt tolerance evaluation results. Recently, relative water content (RWC) has been extensively applied as a criterion for evaluating barley, wheat and rice plant physiological responses to abiotic stresses (Munns et al., 2010). RWC provides an appropriate measure of water content in plant tissue compared to the maximum water carrying capacity of the tissue. RWC is usually used to determine the resistance of plants to salt and drought conditions through comparison of a thermodynamic state, e.g., water potential, turgor potential and solute potential (Sinclair and Ludlo, 1985). In addition, the K^+/Na^+ ratio is one of the key features for plants to show tolerance to salinity (Maathuis and Amtmann, 1999). Under normal condition, plants maintain an optimal K^+/Na^+ ratio that is required for normal plant processes, e.g., enzyme activation, photosynthesis, protein synthesis, stomatal movement, cation-anion balance and stress resistance (Chen et al., 2005). Under excessive salinity levels, plants

take up a high amount of Na^+ while the amount of K^+ uptake is decreased, leading to a non-functioning cell membrane (Ashraf, 2004). The K^+/Na^+ ratio has been used as an important tool for screening plant responses to salt stress in cotton, barley and wheat (Ashraf, 2004). Hence, the relative water content (RWC) and Na^+/K^+ ratio were chosen in this study as being important criteria to consider for assessment of salt tolerance ability.

Ideally, any study of a gene expression response to exposure to acute stress levels is profiled in a time-point range (Seki et al., 2002), while exposure to chronic stress levels is usually used as a way to examine plant exposure to cyclic or continuous mild stress under field conditions (Watkinson et al., 2003). It is difficult, however, to manage the interaction between salt concentration and other environmental factors (Genc et al., 2007). Measurement of responses to exposure to chronic stress conditions is more complicated (Cramer et al., 2011). Chronic stress also negatively impacts upon developmental pathways, leading to inaccurate comparisons being made between the control and stress-exposed samples. Changes in transcript abundance under exposure to acute stress is largely the same as those found in chronic stress exposure situations (Tattersall et al., 2007); hence the application of acute stress conditions is preferred to avoid this complication. Under laboratory conditions, plants exposed to salinity tend to express a rapid and temporary reduction in growth rate, due to reduced water uptake, which results in a slower rate of growth (Munns, 2002). In the present study, 15 barley varieties were screened for their tolerance to acute salt exposure by assessing the key physiological parameters at two different time-points (6 and 24 hours) under 150 mM NaCl condition, the minimum concentration needed to obtain significant differences between genotypes (Rahnama et al., 2010). The results allowed identifying salt tolerant varieties which were used for study of the related salt tolerance genes in the following chapter.

7.2 Effect of salinity on relative water content

7.2.1 Relative water content after 6 hours of salt treatment

One of the parameters used to evaluate the effect of salinity on barley varieties is the shoot relative water content index (RWCI), which describes the changes in RWC after exposure to acute salt stress conditions compared to that of controls in the absence of salt (Kausar et al., 2012). After 6 hours of exposure to 150 mM NaCl, 15 barley

cultivars showed a wide range of responses, expressing RWCI as showed in Figure 7.2.1. Most of the barley cultivars exhibited a reduction in their shoot water content compared to that of the control, whereas some genotypes did not show any change, with RWCI values ranging from 70 to 100%. The most significant reduction was observed in the Dask (RWCI: 71.21%) and Steptoe (73.11%) varieties, while the Mundah, Skiff and Calmariout varieties were found to exhibit almost no change in their RWC after 6 hours of exposure to the salinity conditions (Table 7.2.1).

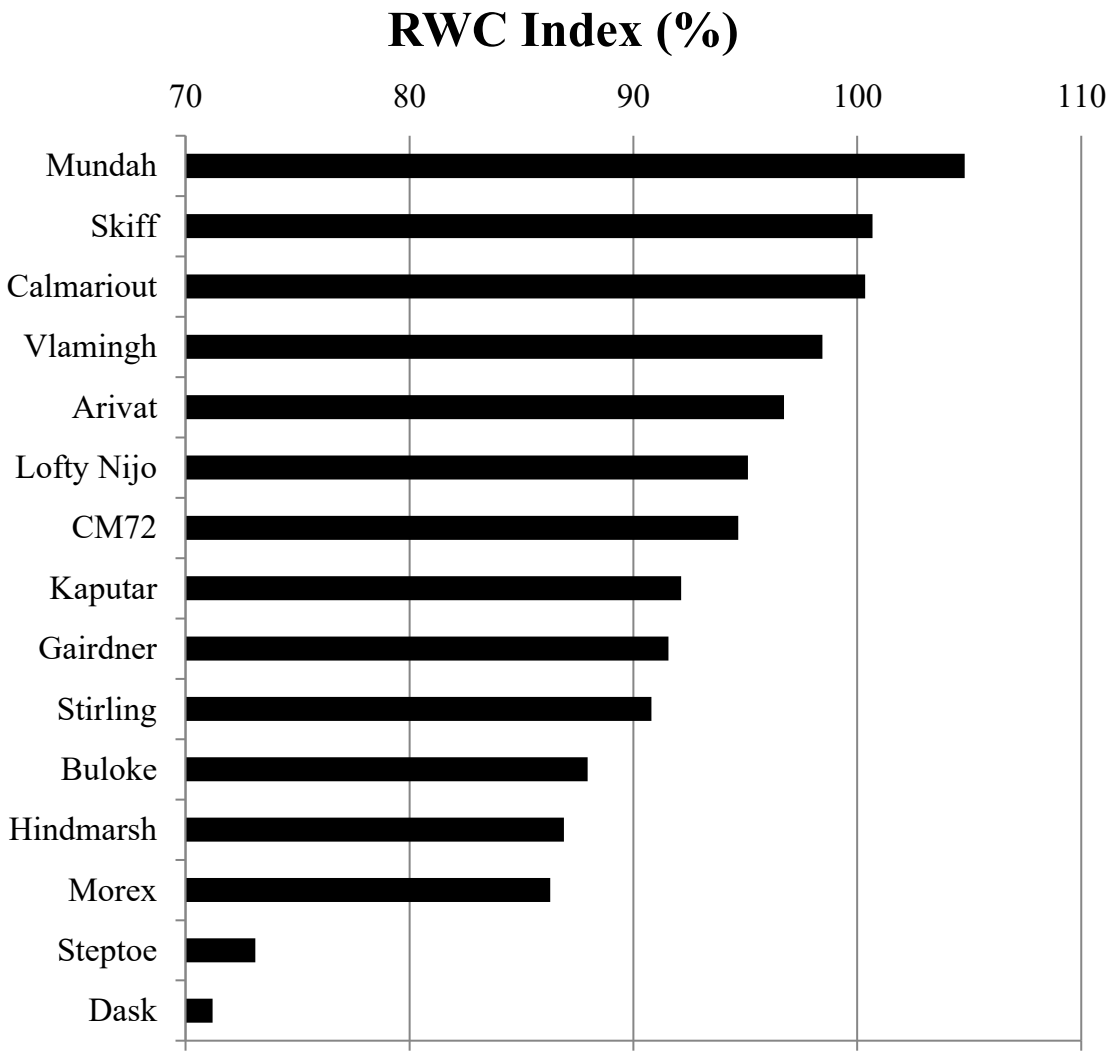

Figure 7.2.1 Effect of salt stress on the RWC index after 6 h of salt exposure.

The relative water content stress tolerance index was calculated as RWCI = (RWC stressed plant/RWC control plant) ×100.

Table 7.2.1 Effects of 6h salinity stress (150 mM NaCl) on plant growth and physiological parameters of 15 varieties of barley.

	Arivat	Buloke	Calmariout	CM72	Dask	Gairdner	Hindmarsh	Kaputar
Shoot length (cm) control	34.8 ± 1.2	32.9 ± 1.9	33.3 ± 2.7	36.8 ± 0.6	37.7 ± 0.8	32.6 ± 1.2	25.2 ± 0.6	33.6 ± 1.6
Shoot length (cm) stress	37.0 ± 0.8	32.7 ± 1.2	37.0 ± 3.2	37.6 ± 1.2	38.0 ± 0.5	34.3 ± 0.8	25.2 ± 1.3	28.8 ± 0.9
Root length (cm) control	10.8 ± 2.0	13.1 ± 0.3	16.9 ± 0.7	16.8 ± 0.7	19.9 ± 1.2	16.5 ± 0.3	14.6 ± 0.7	17.6 ± 1.9
Root length (cm) stress	18.6 ± 1.9	14.0 ± 1.4	15.5 ± 1.1	14.6 ± 0.3	18.4 ± 0.6	14.5 ± 1.2	11.9 ± 0.7	13.8 ± 0.9
Shoot fresh weight (Control)	0.550 ± 0.012	0.613 ± 0.032	0.583 ± 0.028	0.861 ± 0.088	0.893 ± 0.057	0.948 ± 0.116	0.320 ± 0.015	0.636 ± 0.077
Shoot turgid weight (Control)	0.720 ± 0.010	0.733 ± 0.037	0.783 ± 0.037	0.938 ± 0.077	1.114 ± 0.196	0.991 ± 0.121	0.423 ± 0.031	0.810 ± 0.101
Shoot dry weight (Control)	0.040 ± 0.001	0.047 ± 0.005	0.040 ± 0.002	0.114 ± 0.011	0.182 ± 0.036	0.130 ± 0.012	0.028 ± 0.001	0.050 ± 0.005
RWC Control	0.75 ± 0.01	0.82 ± 0.01	0.73 ± 0.02	0.90 ± 0.05	0.80 ± 0.12	0.95 ± 0.03	0.74 ± 0.02	0.77 ± 0.01
Shoot fresh weight (stress)	0.630 ± 0.076	0.470 ± 0.035	0.680 ± 0.113	1.025 ± 0.138	0.683 ± 0.056	0.929 ± 0.054	0.300 ± 0.020	0.420 ± 0.020
Shoot turgid weight (stress)	0.840 ± 0.076	0.630 ± 0.030	0.913 ± 0.150	1.166 ± 0.138	1.091 ± 0.177	1.051 ± 0.056	0.466 ± 0.056	0.576 ± 0.023
Shoot dry weight (stress)	0.046 ± 0.004	0.042 ± 0.003	0.047 ± 0.006	0.147 ± 0.016	0.178 ± 0.024	0.124 ± 0.004	0.028 ± 0.003	0.033 ± 0.001
RWC Stress	0.73 ± 0.02	0.72 ± 0.02	0.73 ± 0.01	0.85 ± 0.02	0.57 ± 0.06	0.87 ± 0.04	0.64 ± 0.06	0.71 ± 0.02
RWC Index	**96.72**	**87.95**	**100.35**	**94.69**	**71.21**	**91.56**	**86.90**	**92.14**
Ranking by RWCI	**5**	**11**	**3**	**7**	**15**	**9**	**12**	**8**

RWC= (FW-DW) / (TW-DW); RWC Index (RWCI) = (RWC (stress)/RWC (control))×100. All data are shown as average of 3 biological replicates, with standard errors.

Table 7.2.1 Continued.

	Lofty Nijo	Morex	Mundah	Skiff	Steptoe	Stirling	Vlamingh
Shoot length (cm) control	37.5 ± 1.0	37.1 ± 0.4	34.9 ± 0.5	36.4 ± 1.2	31.0 ± 2.0	34.3 ± 1.2	26.0 ± 1.5
Shoot length (cm) stress	34.4 ± 2.7	36.3 ± 0.8	36.1 ± 1.1	36.8 ± 1.7	34.4 ± 2.4	31.8 ± 2.5	30.0 ± 1.2
Root length (cm) control	11.5 ± 1.5	14.8 ± 0.7	15.5 ± 2.5	14.5 ± 1.3	13.7 ± 0.5	11.2 ± 0.9	15.0 ± 2.2
Root length (cm) stress	10.6 ±1.1	16.1 ± 1.2	18.9 ± 0.4	18.9 ± 0.8	13.9 ± 1.9	11.2 ± 0.9	14.8 ± 0.9
Shoot fresh weight (Control)	0.510 ± 0.040	1.045 ± 0.038	0.686 ± 0.036	1.316 ± 0.036	0.692 ± 0.103	0.796 ± 0.018	0.440 ± 0.037
Shoot turgid weight (Control)	0.683 ± 0.049	1.042 ± 0.038	0.833 ± 0.063	1.415 ± 0.149	0.845 ± 0.158	0.762 ± 0.078	0.523 ± 0.040
Shoot dry weight (Control)	0.042 ± 0.003	0.148 ± 0.005	0.047 ± 0.003	0.185 ± 0.016	0.140 ± 0.023	0.134 ± 0.013	0.031 ± 0.003
RWC Control	0.72 ± 0.01	1.00 ± 0.02	0.81 ± 0.01	0.93 ± 0.01	0.79 ± 0.04	1.08 ± 0.14	0.82 ± 0.01
Shoot fresh weight (stress)	0.440 ± 0.058	0.892 ± 0.060	0.720 ± 0.032	1.264 ± 0.168	0.770 ± 0.117	0.686 ± 0.014	0.486 ± 0.033
Shoot turgid weight (stress)	0.620 ± 0.076	1.004 ± 0.047	0.833 ± 0.031	1.326 ± 0.180	1.223 ± 0.226	0.693 ± 0.012	0.586 ± 0.033
Shoot dry weight (stress)	0.034 ± 0.004	0.151 ± 0.006	0.048 ± 0.002	0.187 ± 0.031	0.162 ± 0.042	0.151 ± 0.007	0.037 ± 0.001
RWC Stress	0.69 ± 0.05	0.86 ± 0.03	0.85 ± 0.01	0.94 ± 0.02	0.58 ± 0.04	0.98 ± 0.12	0.81 ± 0.01
RWC Index	**95.12**	**86.29**	**104.79**	**100.68**	**73.11**	**90.80**	**98.44**
Ranking by RWCI	**6**	**13**	**1**	**2**	**14**	**10**	**4**

RWC= (FW-DW) / (TW-DW); RWC Index (RWCI) = (RWC (stress)/RWC (control)) ×100. All data are shown as average of 3 biological replicates, with standard errors.

7.2.2 Relative water content after 24 hours of salt treatment

The water-carrying capacity of plant tissue exhibited substantial changes after 24 hours of salt treatment compared to that obtained after 6 hours of salt treatment (Figure 7.2.2). The absorbance of water in the Stirling, Buloke and Steptoe varieties almost reached a saturation level, with only a minor change in their RWC values, while water uptake was substantially decreased in the Morex (RWCI: 70.57%) and Gairdner (RWCI: 76.42%) varieties after 24 hours of salt exposure (Table 7.2.2).

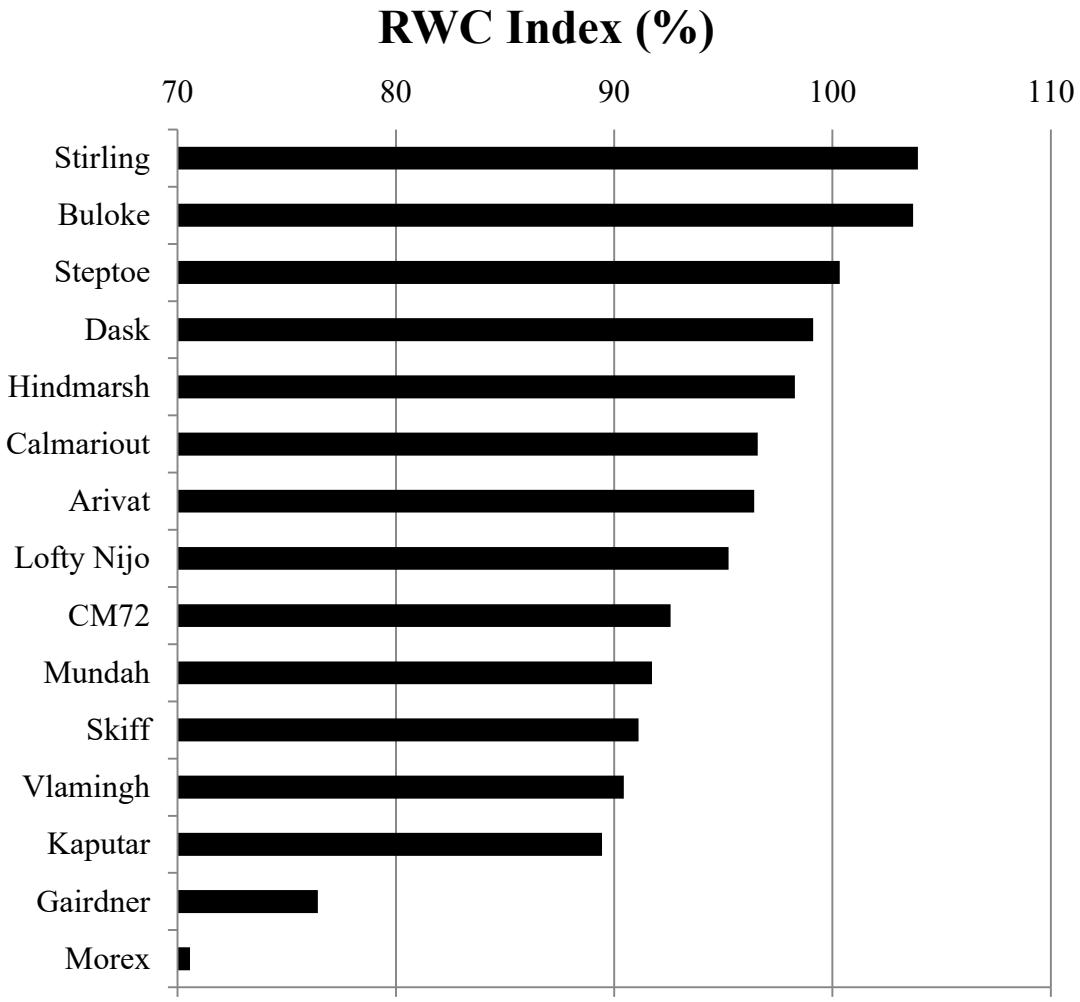

Figure 7.2.2 Effect of salt stress on the RWC index after 24 h of salt exposure.

The relative water content stress tolerance index was calculated as RWCI= (RWC stressed plant/RWC control plant) ×100.

Table 7.2.2 Effects of 24h salinity stress (150 mM NaCl) on plant growth and physiological parameters of 15 varieties of barley.

	Arivat	Buloke	Calmariout	CM72	Dask	Gairdner	Hindmarsh	Kaputar
Shoot length (cm) control	38.0 ± 1.1	34.4 ± 0.9	32.2 ± 1.7	36.5 ± 1.5	38.0 ± 1.0	34.0 ± 0.5	26.1 ± 1.3	29.7 ± 1.0
Shoot length (cm) stress	36.1 ± 1.9	32.8 ± 2.6	32.8 ± 1.5	36.3 ± 0.3	37.5 ± 0.4	33.6 ± 0.4	25.0 ± 1.6	30.7 ± 0.8
Root length (cm) control	10.4 ± 1.4	7.1 ± 0.4	15.6 ± 0.3	19.6 ± 1.2	23.0 ± 1.2	18.0 ± 2.0	14.5 ± 1.1	14.0 ± 1.3
Root length (cm) stress	15.9 ± 0.3	9.4 ± 1.2	14.9 ± 0.9	15.1 ± 0.4	19.2 ± 1.0	16.1 ± 0.1	12.9 ± 2.1	16.5 ± 0.8
Shoot fresh weight (Control)	0.660 ± 0.069	0.486 ± 0.023	0.553 ± 0.024	0.900 ± 0.078	0.972 ± 0.040	1.109 ± 0.096	0.406 ± 0.026	0.403 ± 0.006
Shoot turgid weight (Control)	0.843 ± 0.097	0.690 ± 0.040	0.696 ± 0.027	1.334 ± 0.039	1.229 ± 0.058	1.341 ± 0.102	0.540 ± 0.050	0.473 ± 0.008
Shoot dry weight (Control)	0.050 ± 0.006	0.028 ± 0.004	0.040 ± 0.001	0.099 ± 0.012	0.203 ± 0.002	0.134 ± 0.011	0.034 ± 0.002	0.028 ± 0.001
RWC Control	0.77 ± 0.02	0.69 ± 0.01	0.78 ± 0.01	0.64 ± 0.05	0.75 ± 0.03	0.81 ± 0.06	0.74 ± 0.03	0.84 ± 0.01
Shoot fresh weight (stress)	0.616 ± 0.04	0.493 ± 0.038	0.583 ± 0.049	0.827 ± 0.051	0.767 ± 0.052	1.100 ± 0.078	0.390 ± 0.020	0.480 ± 0.040
Shoot turgid weight (stress)	0.813 ± 0.059	0.676 ± 0.038	0.760 ± 0.065	1.285 ± 0.078	0.986 ± 0.131	1.707 ± 0.114	0.526 ± 0.046	0.626 ± 0.061
Shoot dry weight (stress)	0.048 ± 0.002	0.020 ± 0.002	0.041 ± 0.003	0.143 ± 0.023	0.172 ± 0.034	0.110 ± 0.007	0.033 ± 0.002	0.041 ± 0.002
RWC Stress	0.74 ± 0.01	0.71 ± 0.02	0.75 ± 0.02	0.59 ± 0.01	0.74 ± 0.06	0.61 ± 0.00	0.72 ± 0.03	0.75 ± 0.04
RWC Index	**96.42**	**103.70**	**96.58**	**92.58**	**99.11**	**76.42**	**98.28**	**89.45**
Ranking by RWCI	**7**	**2**	**6**	**9**	**4**	**14**	**5**	**13**

RWC= (FW-DW) / (TW-DW); RWC Index (RWCI) = (RWC (stress)/RWC (control)) ×100. All data are shown as average of 3 biological replicates, with standard errors.

Table 7.2.2 Continued.

	Lofty Nijo	Morex	Mundah	Skiff	Steptoe	Stirling	Vlamingh
Shoot length (cm) control	34.0 ± 0.4	35.7 ± 3.0	38.0 ± 1.6	35.8 ± 1.4	27.2 ± 1.9	29.6 ± 2.1	31.0 ± 1.4
Shoot length (cm) stress	31.6 ± 1.9	33.1 ± 0.9	35.4 ± 1.4	37.4 ± 1.2	34.1 ± 1.3	35.0 ± 0.5	28.0 ± 1.2
Root length (cm) control	13.6 ± 0.6	14.2 ± 0.2	17.5 ± 0.5	13.7 ± 0.8	11.8 ± 0.8	9.9 ± 0.4	12.2 ± 1.8
Root length (cm) stress	14.1 ± 0.9	13.8 ± 1.8	13.1 ± 1.4	17.7 ± 2.6	13.1 ± 0.8	12.1 ± 1.3	15.5 ± 2.3
Shoot fresh weight (Control)	0.650 ± 0.055	1.016 ± 0.168	0.636 ± 0.034	1.288 ± 0.095	0.625 ± 0.021	0.566 ± 0.062	0.486 ± 0.056
Shoot turgid weight (Control)	0.833 ± 0.046	1.443 ± 0.153	0.756 ± 0.046	1.442 ± 0.095	0.712 ± 0.014	0.668 ± 0.065	0.626 ± 0.068
Shoot dry weight (Control)	0.050 ± 0.002	0.089 ±0.019	0.048 ± 0.002	0.120 ± 0.014	0.120 ± 0.009	0.108 ± 0.015	0.036 ± 0.004
RWC Control	0.76 ± 0.04	0.67 ± 0.04	0.83 ± 0.01	0.88 ± 0.02	0.85 ± 0.02	0.81 ± 0.01	0.76 ± 0.01
Shoot fresh weight (stress)	0.483 ± 0.041	0.796 ± 0.023	0.576 ± 0.080	1.134 ± 0.015	0.855 ± 0.210	0.714 ± 0.035	0.473 ± 0.058
Shoot turgid weight (stress)	0.650 ± 0.065	1.595 ± 0.051	0.736 ± 0.078	1.386 ± 0.029	0.974 ± 0.147	0.826 ± 0.054	0.670 ± 0.080
Shoot dry weight (stress)	0.039 ± 0.002	0.070 ± 0.004	0.046 ± 0.005	0.098 ± 0.008	0.143 ± 0.023	0.123 ± 0.011	0.036 ± 0.004
RWC Stress	0.72 ± 0.01	0.47 ± 0.01	0.76 ± 0.03	0.80 ± 0.01	0.85 ± 0.10	0.84 ± 0.07	0.68 ± 0.01
RWC Index	**95.24**	**70.57**	**91.73**	**91.12**	**100.33**	**103.92**	**90.44**
Ranking by RWCI	**8**	**15**	**10**	**11**	**3**	**1**	**12**

RWC= (FW-DW) / (TW-DW); RWC Index (RWCI) = (RWC (stress)/RWC (control)) ×100. All data are shown as average of 3 biological replicates, with standard errors.

7.2.3 Effect of salinity on the relative water content of shoots at the 6 and 24 hour time-points

The relative water content of barley plants showed a diverse response to salinity after 24 hours of salt exposure compared to that obtained after 6 hours exposure, as presented in Figure 7.2.3. Among the tested genotypes, the Stirling, Buloke, Hindmarsh, Steptoe and Dash genotypes exhibited an increase in their RWCI, in which the Stirling, Buloke and Steptoe genotypes reached a saturation point with regard to their capability of retaining water, compared to the control samples in the absence of salt after 24 hours of exposure. The Arivat and Lofty Nijo genotypes showed almost no change in their RWCI, whereas the remaining 8 genotypes decreased in RWCI between 6 and 24 hours stress, with severe drop of RWCI in Morex (from 86.29% to 70.57%) and Gairdner (from 91.56% to 76.42%) (Figure 7.2.3).

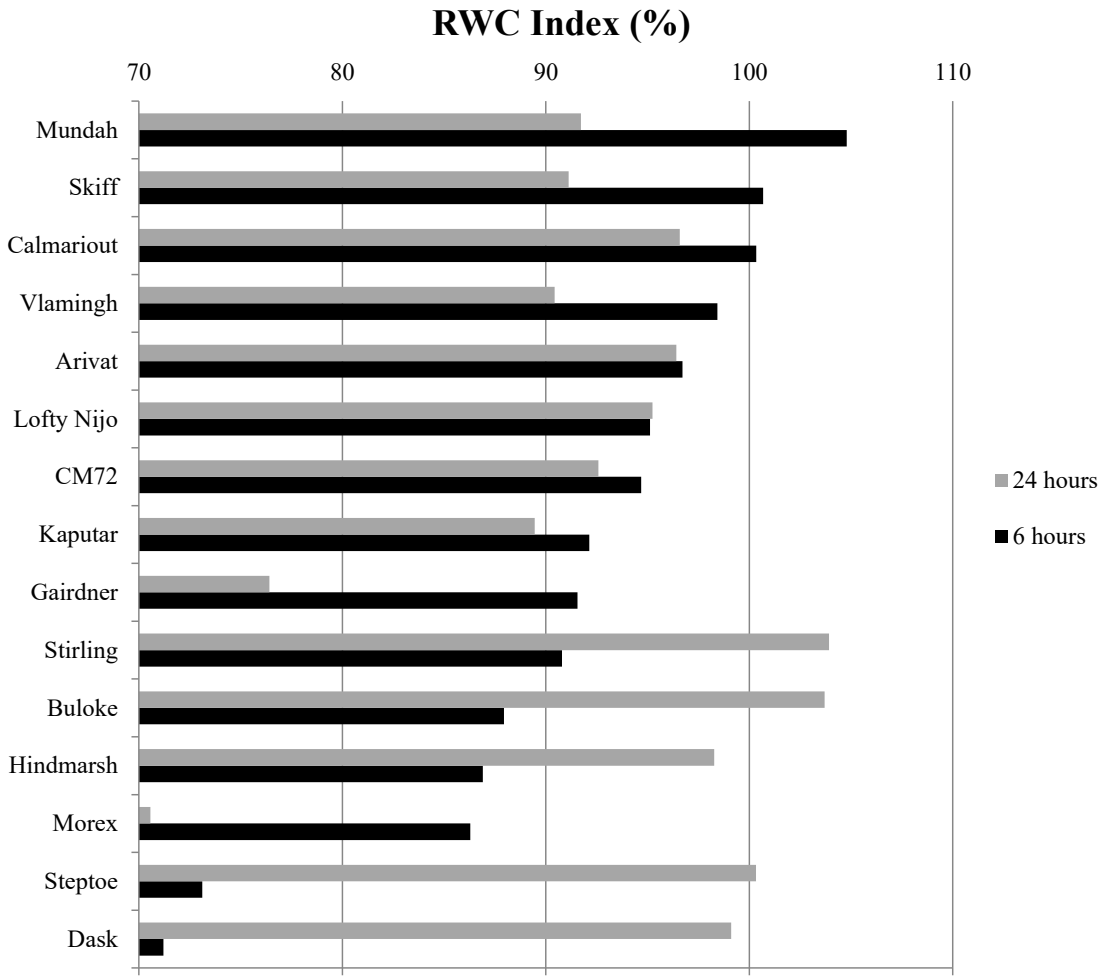

Figure 7.2.3 Effect of salt stress on the RWC index after 6 and 24 hours of salt exposure

The relative water content stress tolerance index was calculated as RWCI= (RWC stressed plant/RWC control plant) ×100.

The RWC of the barley genotypes tested showed a continuous decrease or increase compared to the results published by Kamboj et al. (2015), where the salt tolerant barley genotypes after 12 hours of 150 mM NaCl treatment were identified (Table 7.2.3). A continuous reduction was observed in the RWC of the Calmariout and Mundah genotypes, while the RWC on the Hindmarsh and Buloke genotypes continuously increased with time. The Vlamingh and Gairdner genotypes retained their maximum water content after 12 hours of treatment, with only a slight change in the RWCI being observed after 12 and 24 hours of salt exposure, from 90.55% to 90.44% and 73.55% to 76.42%, respectively. The CM72 genotype showed remained almost constant in its ability to carry water after 6 hours of salt exposure, with only slightly differences in the RWCI

being observed at each time-point. The study of Ahmed et al. (2013) also reported no significant changes in the RWC (RWCI ~95%) of the CM72 genotype under salinity conditions compared to that of the control after 35 days of treatment with 200 mM NaCl. The CM72 genotype may have the ability to retain its maximum water-carrying capacity during the first 6 hours of stress conditions. The Arivat genotype, however, exhibited a significant difference in the RWC under salinity exposure compared to that obtained for the control samples after 12 hours of treatment, whereas no significant changes in RWC were observed after 6 and 24 hours of exposure.

Table 7.2.3 Relative water content index of barley genotypes grown under salinity exposure conditions at different time-points.

Variety	RWCI (%)		
	6 hours	**12 hours**	**24 hours**
Calmariout	100.35	97.22*	96.58
Mundah	104.79	96.05*	91.73
Hindmarsh	86.29	96.29*	98.28
Buloke	87.95	91.84*	103.70
Vlamingh	98.44	90.55*	90.44
Skiff	100.68	86.50*	91.12
CM72	94.69	85.34*	92.58
Arivat	96.72	77.28*	96.42
Gairdner	91.56	73.55*	76.42

*: data from Kamboj et al. (2015)

7.3 Effect of salinity on the Na^+/K^+ ratio

7.3.1 Na^+/K^+ ratio in plant tissue after 6 hours of exposure to salinity conditions

The ability of retaining the $[K^+]$ loss and preventing $[Na^+]$ uptake from plants is another criterion for evaluating levels of salt tolerance (Ahmed et al., 2013). The sodium concentration was found to be increased in all genotypes as a function of time, accumulating in the shoots more so than the root after 6 hours of salt exposure. The $[Na^+]$ ranged from 4.11 to 9.12 mg/g dry weight (DW) in the shoots and from 4.40 to 8.91 mg/g DW in the roots under normal conditions, increasing to 21.16 to 71.78 mg/g DW in the shoots and from 14.18 to 39.27 mg/g DW in the roots of stressed plants (Table 7.3.1;

Figure 7.3.1). The greatest increase in the shoot [Na^+] index was observed in the Buloke genotype (1325.10%), followed by that of the CM72 (1205.89%) and Skiff (1118.12%) genotypes, while the Mundah genotype was found to be the least impacted plant, with a 484.54% increase in [Na^+] being observed. There was a significant increase in the [Na^+] index, with increases between 484.54-1325.10% being measured for the shoots, but not for the roots (215.43-606.12%). Conversely, the [K^+] decreased under salt stress conditions, from 51.33 - 65.66 mg/g to 36.78 - 48.72 mg/g DW in the shoots and 9.05 - 14.70 mg/g to 5.91 - 9.90 mg/g DW in the roots of the control samples after 6 hours of exposure (Table 7.3.1; Figure 7.3.2). The Stirling and Mundah genotypes exhibited the maximum decrease in the shoot (86.67%) and root (96.27%) [K^+] index, respectively, whereas the lowest decreases were observed in the Arivat genotype shoot (61.25%) and Dask genotype root (48.16%) [K^+] index. The Na^+/K^+ ratio was more affected in the shoots compared to that of the roots; under normal conditions, the Na^+/K^+ ratio ranged from 0.07 - 0.17 in the shoots and from 0.33 - 0.99 in the roots. After 6 hours of salt exposure, the ratio rapidly increased, with the highest Na^+/K^+ ratio index being observed in the Buloke genotype shoots and CM72 genotype roots, whereas the lowest Na^+/K^+ ratio index was measured for the Hindmarsh genotype shoots and Mundah genotype roots (Table 7.3.1; Figure 7.3.3).

Table 7.3.1 Effects of 6h salinity stress (150 mM NaCl) on Na^+/K^+ ion ratios in 15 varieties of barley.

	Arivat	Buloke	Calmariout	CM72	Dask	Gairdner	Hindmarsh	Kaputar
Na^+ control (shoot)	4.42 ± 0.24	4.88 ± 0.20	6.48 ± 0.26	4.86 ± 0.19	6.95 ± 1.03	5.17 ± 0.13	4.11 ± 1.18	5.61 ± 0.30
Na^+ stress (shoot)	44.96 ± 2.49	64.75 ± 10.34	55.95 ± 8.54	58.68 ± 6.76	58.06 ± 4.93	41.32 ± 5.79	21.16 ± 11.20	41.03 ± 2.81
Na^+ Index (shoot)	1017.27	1325.10	862.64	1205.89	835.44	798.26	514.42	736.24
Na^+ control (root)	4.81 ± 0.29	6.02 ± 0.99	7.59 ± 0.90	5.01 ± 0.66	6.58 ± 0.22	5.1 ± 0.41	8.91 ± 0.67	6.42 ± 0.44
Na^+ stress (root)	14.18 ± 2.65	22.44 ± 1.83	15.97 ± 1.53	20.70 ± 2.36	16.97 ± 0.06	22.71 ± 1.98	19.21 ± 3.36	27.37 ± 3.02
Na^+ Index (root)	294.59	372.81	210.27	413.03	257.82	442.75	215.44	425.98
K^+ control (shoot)	60.04 ± 3.70	62.89 ± 3.71	54.72 ± 1.98	53.68 ± 3.37	61.12 ± 2.63	51.33 ± 3.82	55.58 ± 5.08	63.75 ± 2.19
K^+ stress (shoot)	36.78 ± 0.80	47.84 ± 2.60	45.67 ± 2.01	44.5 ± 0.18	46.14 ± 1.22	40.47 ± 0.57	47.76 ± 1.46	42.42 ± 2.12
K^+ Index (shoot)	61.25	76.08	83.49	82.98	75.48	78.83	85.93	66.54
K^+ control (root)	13.16 ± 0.23	9.29 ± 0.72	9.13 ± 0.40	14.7 ± 0.63	12.27 ± 0.64	12.79 ± 3.53	9.05 ± 0.58	10.53 ± 0.32
K^+ stress (root)	7.05 ± 0.06	7.07 ± 0.92	7.87 ± 0.54	7.73 ± 0.48	5.91 ± 0.66	8.37 ± 0.46	8.19 ± 1.26	6.95 ± 1.17
K^+ Index (root)	53.60	76.08	86.27	52.64	48.16	65.51	90.53	66.03
Na^+/K^+ control (shoot)	0.07	0.08	0.11	0.09	0.11	0.10	0.07	0.08
Na^+/K^+ stress (shoot)	1.22	1.36	1.22	1.31	1.26	1.01	0.43	0.98
Na^+/K^+ Index (shoot)	**1643.43**	**1733.84**	**1026.15**	**1441.53**	**1093.72**	**995.14**	**606.18**	**1118.70**
Na^+/K^+ control (root)	0.36	0.67	0.82	0.33	0.53	0.46	0.99	0.61
Na^+/K^+ stress (root)	2.01	3.31	2.06	2.68	2.93	2.70	2.63	4.04
Na^+/K^+ Index (root)	**551.80**	**492.99**	**250.46**	**792.15**	**546.48**	**576.54**	**263.91**	**663.08**
Average Na^+/K^+ Index (root + shoot)	**1097.62**	**1113.42**	**638.31**	**1116.84**	**820.10**	**785.84**	**435.04**	**890.89**
Ranking	**13**	**14**	**4**	**15**	**8**	**7**	**1**	**10**

Na^+ ion Index = (Na^+ (stress)/Na^+ (control)) × 100; Na^+/K^+ ion Index = (Na^+/K^+ (stress)/Na^+/K^+ (control)) × 100

	Lofty Nijo	Morex	Mundah	Skiff	Steptoe	Stirling	Vlamingh
Na^+ control (shoot)	4.88 ± 0.48	6.87 ± 0.83	9.12 ± 1.49	6.42 ± 0.67	7.18 ± 1.51	6.01 ± 0.20	6.17 ± 1.45
Na^+ stress (shoot)	48.41 ± 1.24	48.58 ± 3.88	44.20 ± 1.11	71.78 ± 13.05	59.10 ± 4.62	48.04 ± 1.54	60.02 ± 6.85
Na^+ Index (shoot)	991.33	706.44	484.54	1118.12	822.78	798.55	972.24
Na^+ control (root)	6.48 ± 2.32	8.45 ± 2.39	8.42 ± 0.77	6.12 ± 0.24	6.70 ± 0.76	5.17 ± 0.56	4.40 ± 0.79
Na^+ stress (root)	39.27 ± 11.73	21.32 ± 2.35	19.10 ± 1.97	26.98 ± 6.29	21.37 ± 0.65	20.23 ± 2.09	22.81 ± 1.57
Na^+ Index (root)	606.12	252.15	226.88	440.37	318.79	391.36	517.77
K^+ control (shoot)	59.42 ± 1.54	65.66 ± 2.20	54.40 ± 4.51	55.43 ± 2.01	59.75 ± 4.12	53.87 ± 3.16	57.43 ± 2.55
K^+ stress (shoot)	46.58 ± 2.02	48.72 ± 0.45	38.47 ± 0.24	43.92 ± 2.19	47.04 ± 4.05	46.69 ± 1.55	42.27 ± 1.47
K^+ Index (shoot)	78.38	74.19	70.67	79.22	78.71	86.67	73.60
K^+ control (root)	13.37 ± 1.72	9.39 ± 0.93	10.30 ± 1.03	11.00 ± 1.00	12.18 ± 0.64	11.88 ± 0.96	10.91 ± 3.40
K^+ stress (root)	8.85 ± 1.27	7.51 ± 1.20	9.90 ± 1.70	8.07 ± 1.25	8.98 ± 1.12	7.90 ± 0.45	9.58 ± 0.95
K^+ Index (root)	66.17	79.97	96.27	73.36	73.74	66.76	87.81
Na^+/K^+ control (shoot)	0.08	0.10	0.17	0.11	0.12	0.11	0.10
Na^+/K^+ stress (shoot)	1.04	0.99	1.14	1.67	1.27	1.02	1.43
Na^+/K^+ Index (shoot)	**1264.52**	**957.71**	**659.75**	**1428.35**	**1025.09**	**916.59**	**1332.07**
Na^+/K^+ control (root)	0.53	0.97	0.83	0.56	0.54	0.44	0.61
Na^+/K^+ stress (root)	4.20	3.06	1.97	3.48	2.44	2.54	2.39
Na^+/K^+ Index (root)	**778.87**	**314.46**	**236.19**	**620.45**	**446.52**	**568.17**	**389.49**
Average Na^+/K^+ Index (root + shoot)	**1021.69**	**636.09**	**447.96**	**1024.41**	**735.81**	**742.38**	**860.78**
Ranking	**11**	**3**	**2**	**12**	**5**	**6**	**9**

Na^+ ion Index = (Na^+ (stress)/Na^+ (control)) × 100; Na^+/K^+ ion Index = (Na^+/K^+ (stress)/Na^+/K^+ (control)) × 100

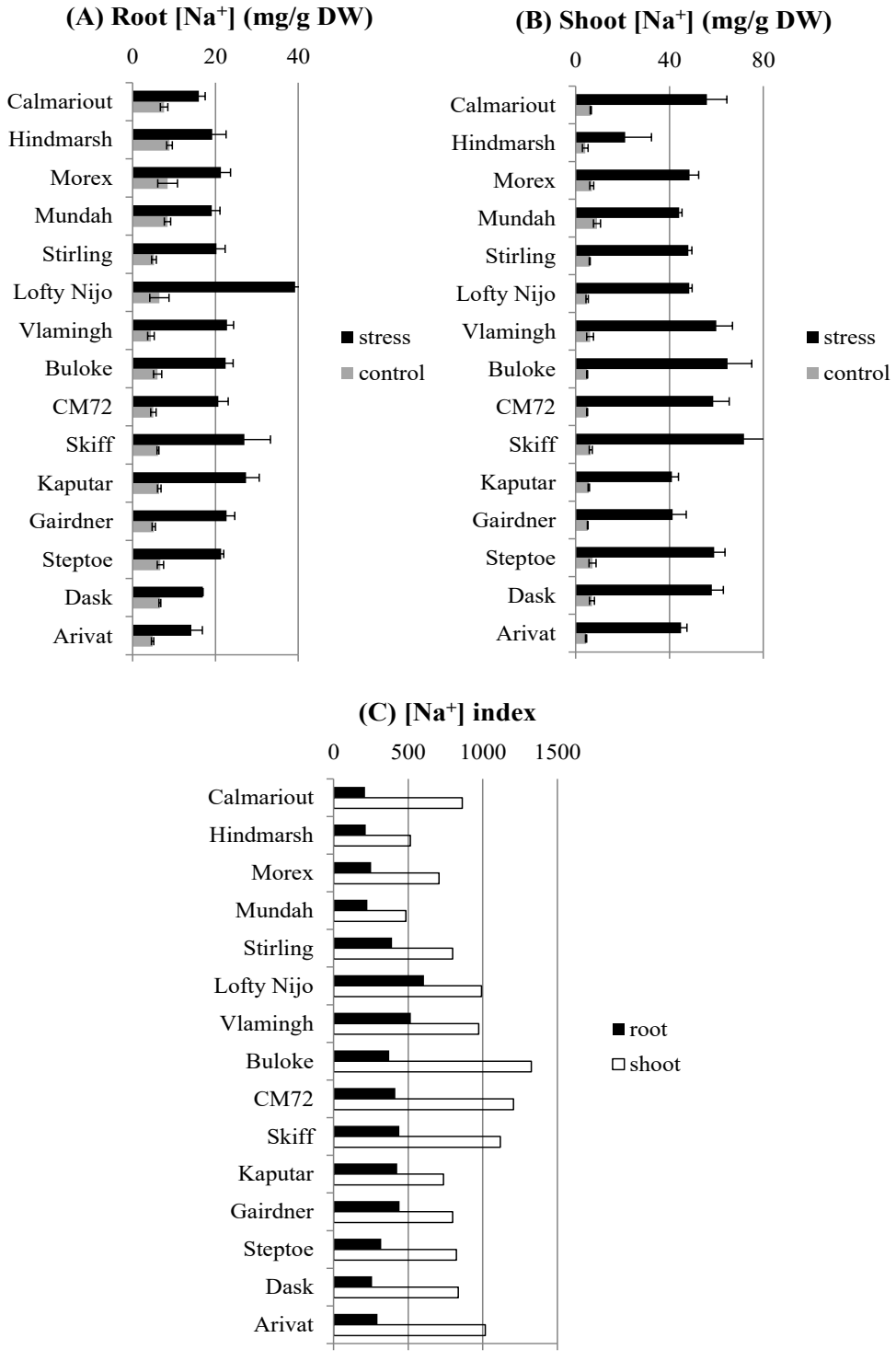

Figure 7.3.1 Effects of 6h salt stress on the [Na^+] and [Na^+] Index.

(A) Uptake of [Na^+] in roots under salt stress conditions; **(B)** Uptake of [Na^+] in shoots under salt stress conditions; **(C)** [Na^+] Index after salt treatment; [Na^+] stress tolerance index as NaI= ([Na^+] stressed plant / [Na^+] control plant) × 100. The error bars represent the standard deviation of three biological replicates.

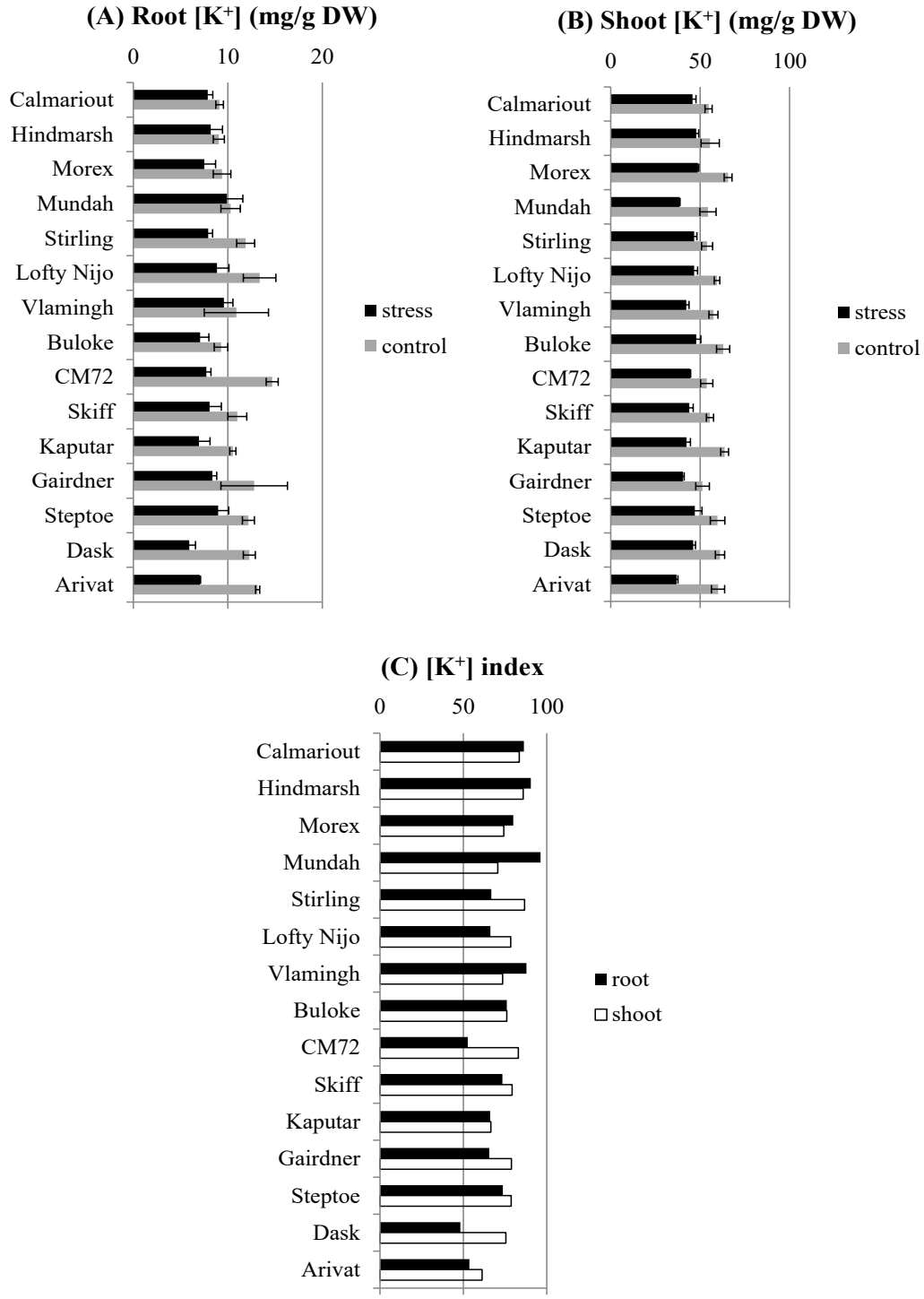

Figure 7.3.2 Effects of 6 h salt stress on the $[K^+]$ and $[K^+]$ Index.

(A) Loss of $[K^+]$ in roots under salt stress conditions; **(B)** Loss of $[K^+]$ in shoots under salt stress conditions; **(C)** $[K^+]$ Index after salt treatment; $[K^+]$ stress tolerance index as KI = ($[K^+]$ stressed plant / $[K^+]$ control plant) × 100. The error bars represent the standard deviation of three biological replicates.

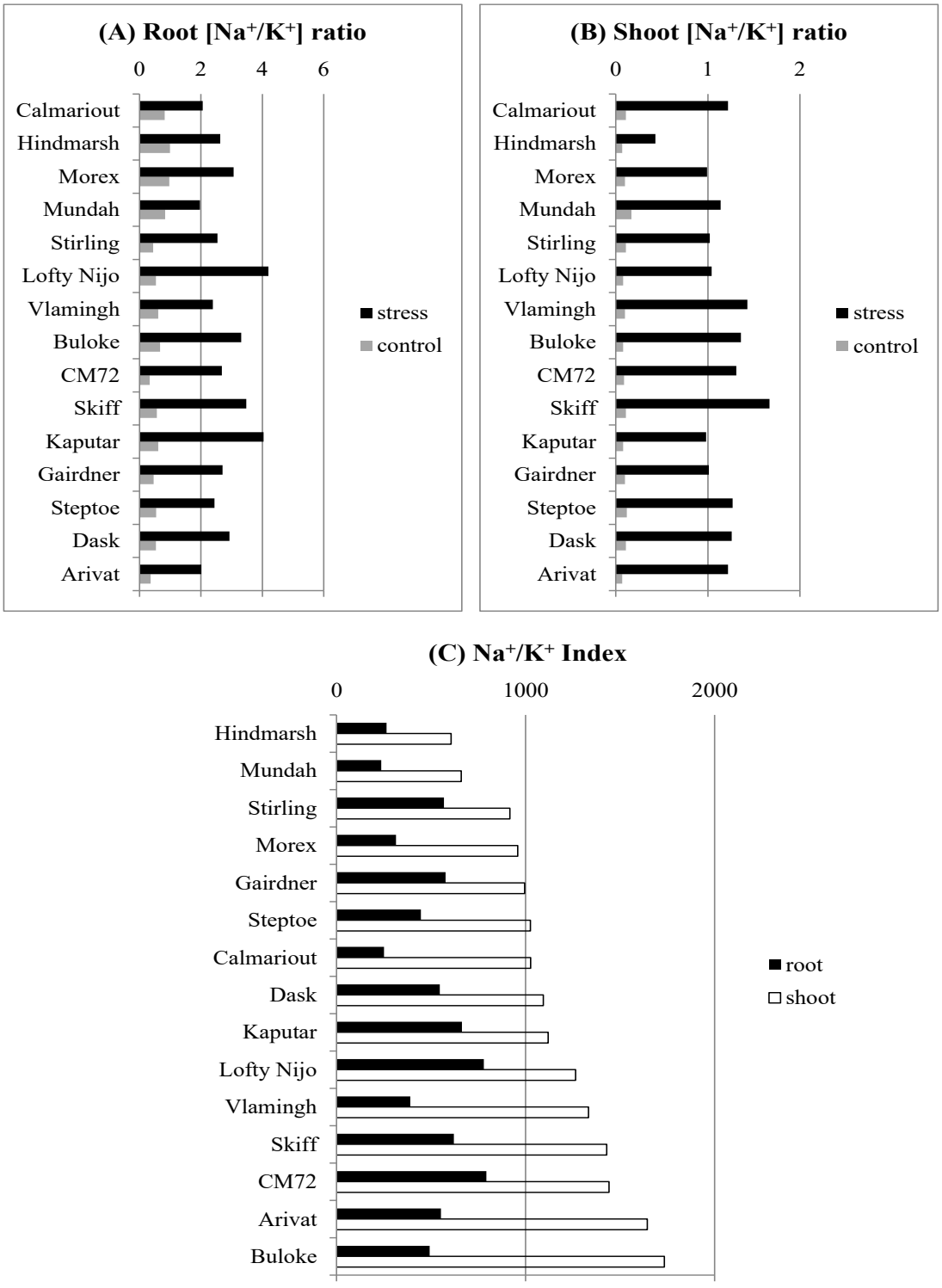

Figure 7.3.3 Effects of 6 h salt stress on the Na^+/K^+ ion ratio index

(A) Na^+/K^+ ratio in roots under salt stress conditions; **(B)** Na^+/K^+ ratio in shoots under salt stress conditions; **(C)** Na^+/K^+ ratio Index after salt treatment; Na^+/K^+ ion stress tolerance index as Na/KI = (Na^+/K^+ ion ratio stressed plant / Na^+/K^+ ratio control plant) × 100.

7.3.2 Na^+/K^+ ratio in plant tissue after 24 hours of exposure to salinity conditions

After 24 h of treatment, all varieties showed a continuous increase in the [Na^+] and decrease in [K^+]. [Na^+] was almost the same as that of the control after 6 h of salt stress exposure, ranging from 4.23 - 8.11 mg/g DW in the shoots and from 4.09 - 7.73 mg/g DW in the roots. The [Na^+] found in the plant tissues exposed to salt was much higher, ranging from 43.39 - 81.17 mg/g DW in the shoots and 20.61 - 38.72 mg/g DW in the roots, compared to those obtained after 6 h of salt exposure (Table 7.3.2; Figure 7.3.4). The maximum increase in the [Na^+] index was found in both the shoot and the root of the Gairdner genotype (1448.03% and 682.77%, respectively), whereas the least change was observed for the shoot of the Skiff (794.05%) and the root of the Buloke (288.87%) genotypes. The [K^+] was reduced from 47.15 - 64.89 mg/g to 30.14 - 43.36 mg/g DW in the shoots and from 8.99 - 15.05 mg/g to 5.28 - 9.58 mg/g DW in the roots. The greatest decrease in the [K^+] index was found in the shoots of the Stirling genotype (85.99%) and in the roots of the Hindmarsh genotype (81.77%); while the least change was observed in the shoots of the Steptoe genotype (50.45%) and the roots of the CM72 genotype (42.94%) (Table 7.3.2; Figure 7.3.5). The highest Na^+/K^+ index ratio in the shoots and roots was observed for the Gairdner genotype (2169.99% in the shoots and 1546.8% in the roots), while the Stirling and Mundah genotypes showed the lowest Na^+/K^+ index ratio in the shoots (1029.35%) and roots (479.25%), respectively (Table 7.3.2; Figure 7.3.6).

Table 7.3.2 Effects of 24h salinity stress (150 mM NaCl) on Na^+/K^+ ion ratios in 15 varieties of barley.

	Arivat	Buloke	Calmariout	CM72	Dask	Gairdner	Hindmarsh	Kaputar
Na^+ control (shoot)	6.33 ± 0.97	5.63 ± 0.17	4.23 ± 0.96	4.28 ± 0.53	4.72 ± 0.39	4.56 ± 0.30	6.13 ± 0.31	8.11 ± 1.71
Na^+ stress (shoot)	57.57 ± 1.11	62.25 ± 1.25	43.56 ± 8.14	55.12 ± 4.90	44.82 ± 14.72	66.03 ± 2.10	79.01 ± 9.43	81.17 ± 11.53
Na^+ Index (shoot)	908.89	1104.37	1029.78	1287.85	948.24	1448.03	1290.41	1000.90
Na^+ control (root)	6.42 ± 0.62	7.73 ± 0.58	6.33 ± 1.81	4.47 ± 1.06	6.34 ± 0.03	5.63 ± 0.07	6.08 ± 0.44	6.01 ± 1.07
Na^+ stress (root)	38.72 ± 4.87	22.34 ± 4.53	24.62 ± 2.02	23.95 ± 3.81	27.66 ± 1.81	38.44 ± 2.54	20.61 ± 2.40	21.16 ± 8.48
Na^+ Index (root)	602.48	288.87	388.99	535.14	436. 05	682.77	338.98	352.33
K^+ control (shoot)	56.00 ± 0.69	58.66 ± 1.04	53.35 ± 2.82	53.65 ± 10.78	63.34 ± 1.11	47.15 ± 4.59	55.58 ± 1.70	64.89 ± 0.63
K^+ stress (shoot)	31.81 ± 4.70	39.76 ± 4.43	39.76 ± 1.71	37.56 ± 4.41	37.49 ± 1.42	31.31 ± 2.62	41.62 ± 3.42	39.77 ± 3.51
K^+ Index (shoot)	56.81	67.79	74.52	70.01	59.20	66.40	74.91	61.29
K^+ control (root)	13.69 ± 0.52	9.54 ± 1.41	9.54 ± 1.55	15.05 ± 1.27	12.71 ± 0.75	14.43 ± 1.17	8.99 ± 1.75	9.01 ± 0.52
K^+ stress (root)	6.42 ± 0.59	6.75 ± 2.05	7.32 ± 1.15	6.47 ± 0.10	5.73 ± 1.10	6.29 ± 0.45	7.34 ± 2.26	5.28 ± 0.28
K^+ Index (root)	46.88	70.77	76.76	42.94	45.11	43.88	81.77	58.55
Na^+/K^+ control (shoot)	0.11	0.09	0.08	0.08	0.07	0.09	0.11	0.12
Na^+/K^+ stress (shoot)	1.87	1.61	1.08	1.51	1.19	2.13	1.95	2.07
Na^+/K^+ Index (shoot)	**1649.72**	**1675.39**	**1337.30**	**1847.65**	**1607.41**	**2169.99**	**1765.89**	**1653.34**
Na^+/K^+ control (root)	0.46	0.83	0.70	0.29	0.50	0.39	0.73	0.66
Na^+/K^+ stress (root)	6.26	5.18	3.63	3.68	5.21	6.16	4.20	3.86
Na^+/K^+ Index (root)	**1342.13**	**623.54**	**513.66**	**1270.79**	**1038.85**	**1546.80**	**571.12**	**577.78**
Average Na^+/K^+ Index (root + shoot)	**1495.93**	**1149.46**	**925.48**	**1559.22**	**1323.14**	**1858.40**	**1168.51**	**1115.56**
Ranking	**13**	**8**	**1**	**14**	**11**	**15**	**9**	**6**

Na^+ ion Index = (Na^+ (stress)/Na^+ (control)) × 100; Na^+/K^+ ion Index = (Na^+/K^+ (stress)/Na^+/K^+ (control)) × 100

	Lofty Nijo	Morex	Mundah	Skiff	Steptoe	Stirling	Vlamingh
Na^+ control (shoot)	6.88 ± 0.51	5.61 ±0.19	5.58 ± 0.19	6.16 ± 0.48	5.54 ± 0.27	4.92 ± 0.46	5.53 ± 0.85
Na^+ stress (shoot)	62.45 ± 1.51	59.65 ± 4.99	64.09 ± 6.43	48.91 ± 11.50	56.46 ± 1.07	43.39 ± 8.05	64.76 ± 7.63
Na^+ Index (shoot)	907.75	1063.33	1146.45	794.05	1017.97	880.71	1170.36
Na^+ control (root)	6.31 ± 1.10	5.87 ± 0.38	7.55 ± 1.67	5.54 ± 0.43	5.55 ± 0.67	4.09 ± 1.18	6.37 ± 0.37
Na^+ stress (root)	27.74 ± 2.48	26.17 ± 2.13	23.48 ± 0.85	36.64 ± 7.05	22.97 ± 7.98	24.93 ± 2.41	32.55 ± 2.20
Na^+ Index (root)	439.49	445.38	310.90	660.69	413.75	608.62	510.77
K^+ control (shoot)	59.64 ± 7.13	50.82 ± 4.44	53.84 ± 2.39	51.08 ± 1.75	59.74 ± 3.61	50.43 ± 4.28	55.38 ± 2.00
K^+ stress (shoot)	40.71 ± 3.96	33.10 ± 4.47	38.31 ± 2.72	37.65 ± 1.03	30.14 ± 2.53	43.36 ± 0.46	38.01 ± 5.05
K^+ Index (shoot)	68.25	65.12	71.16	73.71	50.45	85.99	68.64
K^+ control (root)	11.99 ± 3.32	10.17 ± 0.90	9.27 ± 0.46	12.98 ± 0.54	12.92 ± 0.39	12.04 ± 1.98	14.50 ± 0.88
K^+ stress (root)	7.31 ± 0.35	7.64 ± 0.58	6.01 ± 0.53	7.39 ± 0.29	7.51 ± 0.32	7.55 ± 0.29	9.58 ± 0.51
K^+ Index (root)	60.96	75.15	64.90	56.95	58.16	62.70	66.03
Na^+/K^+ control (shoot)	0.11	0.11	0.10	0.12	0.09	0.09	0.10
Na^+/K^+ stress (shoot)	1.55	1.89	1.70	1.29	1.90	1.00	1.77
Na^+/K^+ Index (shoot)	**1301.73**	**1688.37**	**1632.45**	**1069.75**	**2031.26**	**1029.35**	**1748.91**
Na^+/K^+ control (root)	0.64	0.58	0.82	0.42	0.43	0.35	0.44
Na^+/K^+ stress (root)	3.78	3.45	3.95	4.90	3.15	3.32	3.42
Na^+/K^+ Index (root)	**585.65**	**589.57**	**479.25**	**1148.85**	**729.95**	**946.18**	**773.73**
Average Na^+/K^+ Index (root + shoot)	**943.69**	**1138.97**	**1055.85**	**1109.30**	**1380.60**	**987.77**	**1261.32**
Ranking	**2**	**7**	**4**	**5**	**12**	**3**	**10**

Na^+ ion Index = (Na^+ (stress)/Na^+ (control)) × 100; Na^+/K^+ ion Index = (Na^+/K^+ (stress)/Na^+/K^+ (control)) × 100

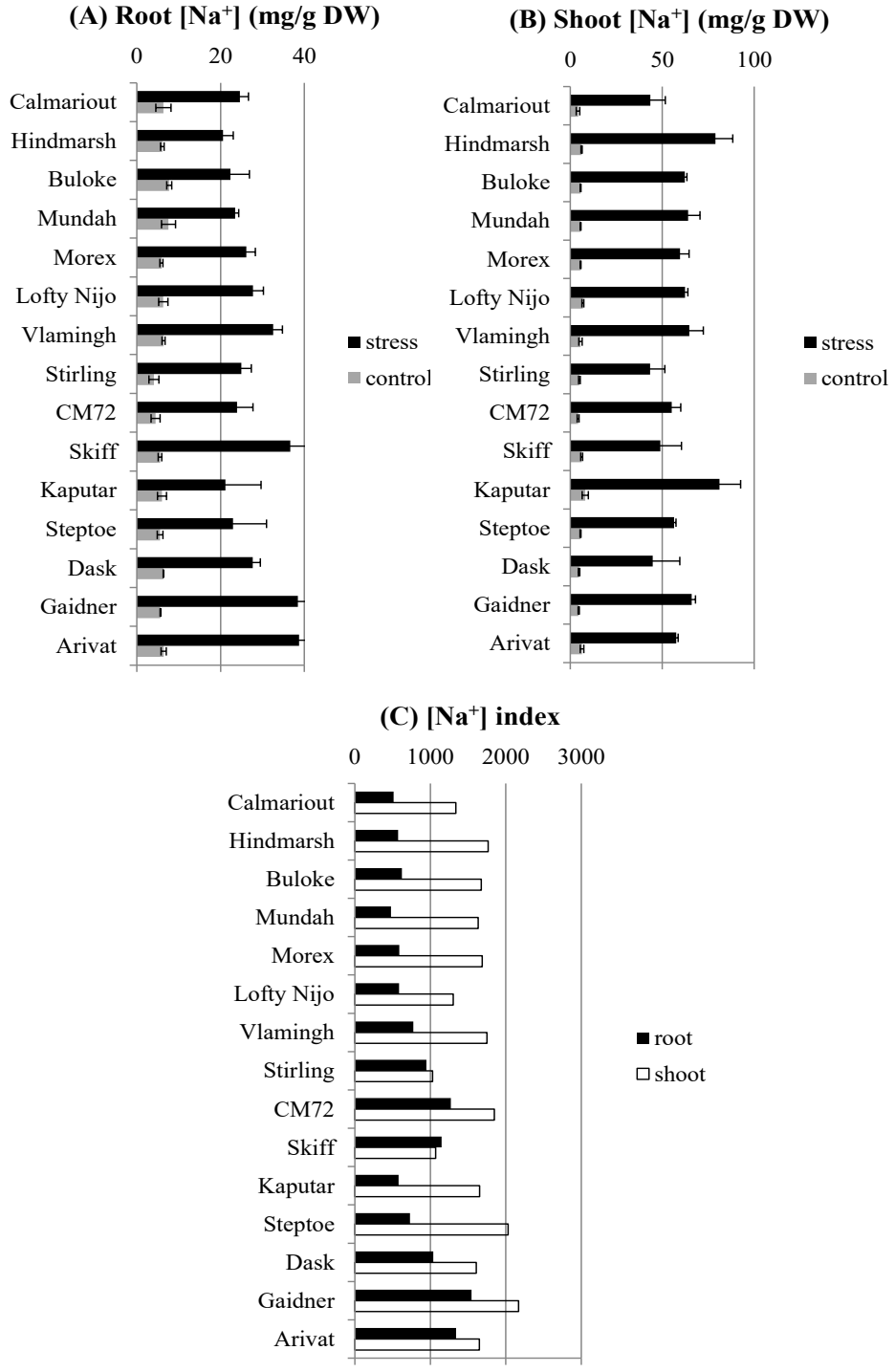

Figure 7.3.4 Effects of 24 h salt stress on the [Na^+] and [Na^+] index.
(A) Uptake of [Na^+] in roots under salt stress conditions; **(B)** Uptake of [Na^+] in shoots under salt stress conditions; **(C)** [Na^+] Index after salt treatment; [Na^+] stress tolerance index as NaI= ([Na^+] stressed plant / [Na^+] control plant) × 100. The error bars represent the standard deviation of three biological replicates.

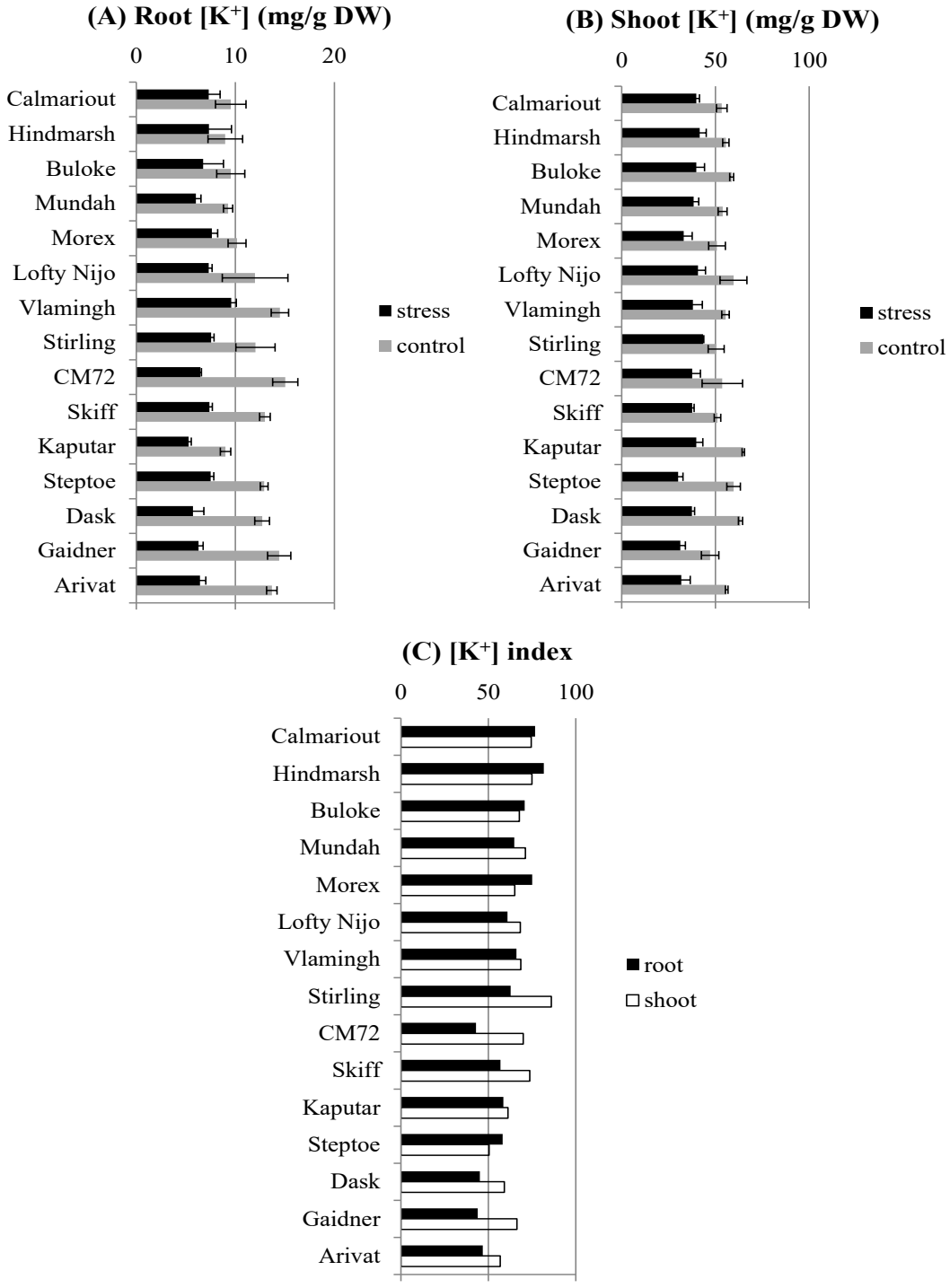

Figure 7.3.5 Effects of 24 h salt stress on the [K^+] and [K^+] index.

(A) Loss of [K^+] in roots under salt stress conditions **(B)** Loss of [K^+] in shoots under salt stress conditions; **(C)** [K^+] Index after salt treatment; [K^+] stress tolerance index as KI = ([K^+] stressed plant / [K^+] control plant) × 100. The error bars represent the standard deviation of three biological replicates.

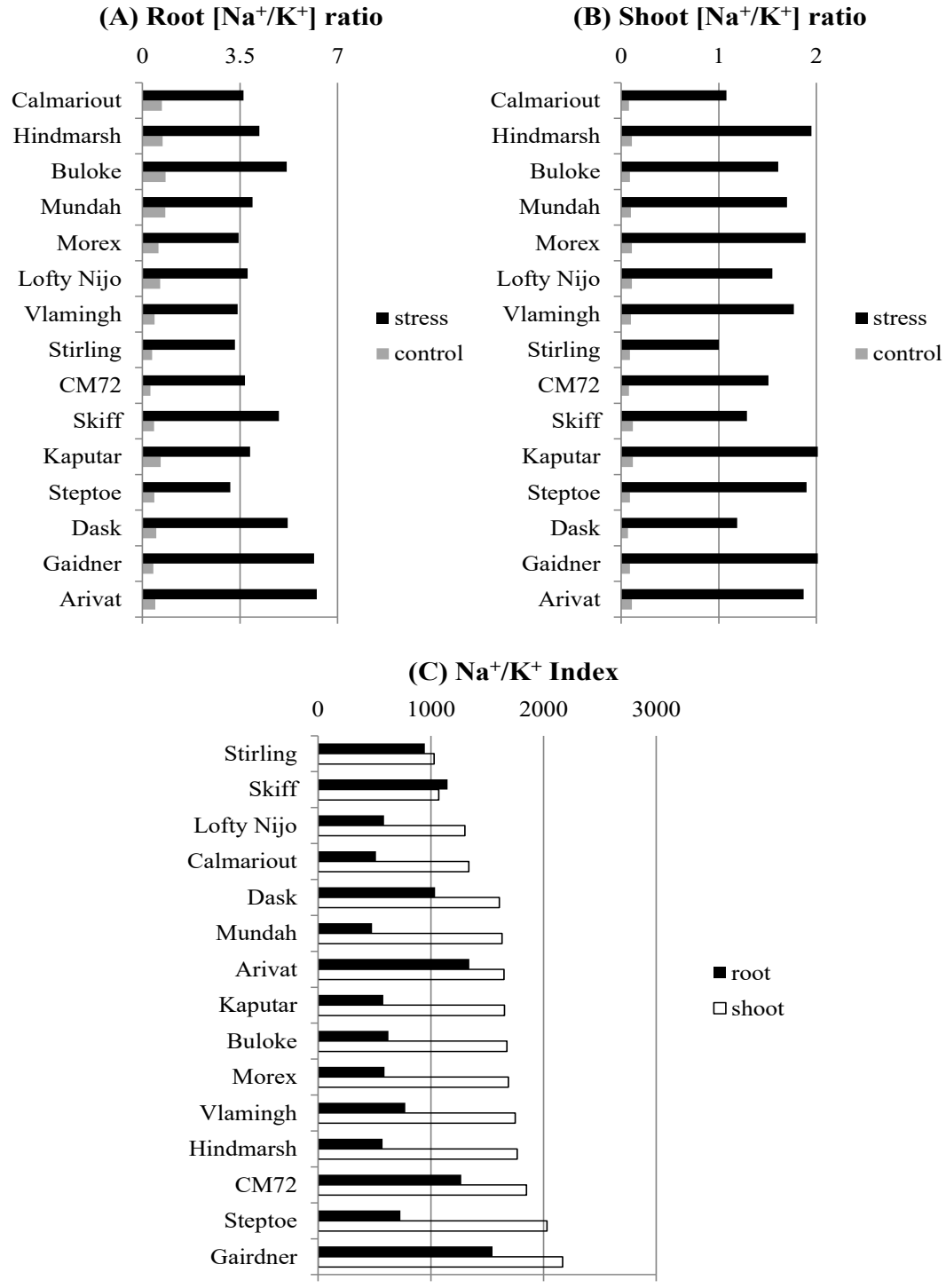

Figure 7.3.6 Effects of 24 h salt stress on the Na^+/K^+ ion ratio index.

(A) Na^+/K^+ ratio in roots under salt stress conditions; **(B)** Na^+/K^+ ratio in shoots under salt stress conditions; **(C)** Na^+/K^+ ratio Index after salt treatment; Na^+/K^+ ion stress

tolerance index as Na/KI = (Na^+/K^+ ion ratio stressed plant / Na^+/K^+ ratio control plant) × 100.

7.3.3 Effect of salinity on Na^+/K^+ ratio in plant tissue at the 6 and 24 hour time-points

In general, Na^+/K^+ ratio index showed continuous increase in both the shoot and the root tissue after 24 hours exposure in compared to 6 hours, except shoot Skiff, shoot Buloke, root Kaputar and root Lofty Nijo with a decrease in their Na^+/K^+ ratio index (Figure 7.3.7). Among the tested genotypes, Gairdner exhibited significant increase of Na^+/K^+ ratio index in both shoot and root. Arivat showed an increase of Na^+/K^+ ratio index in root; however, Na^+/K^+ ratio index unchanged in shoot of this cultivar between 6 h and 24 h exposure to salinity (Figure 7.3.7).

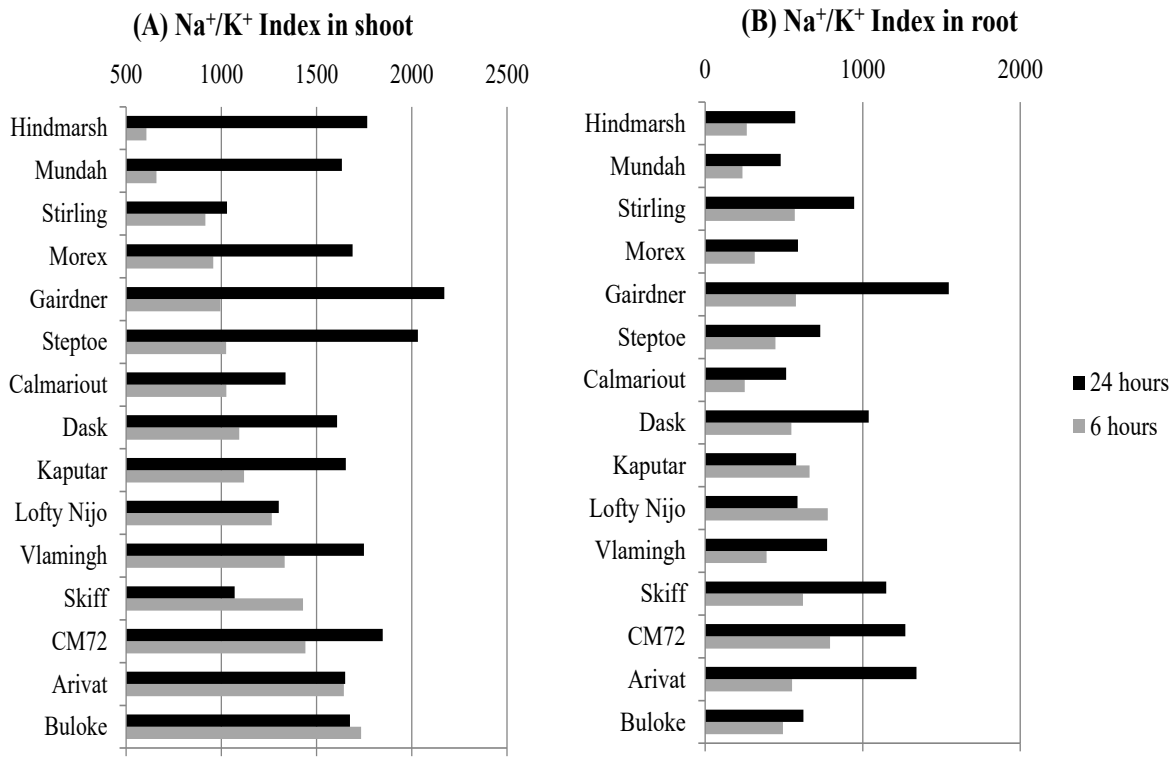

Figure 7.3.7 Na^+/K^+ ion ratio index on plant tissue after 6h and 24h of salt stress conditions.

(A) Na^+/K^+ ratio index in root after 6h and 24h of salt stress conditions; **(B)** Na^+/K^+ ratio index in shoot after 6h and 24h of salt stress conditions; Na^+/K^+ ion stress tolerance index as Na/KI = (Na^+/K^+ ion ratio stressed plant / Na^+/K^+ ratio control plant) × 100.

In compared to data generated by Kamboj et al. (2015), most of the barley plants showed a continuous increase in their Na^+/K^+ ratio index in both their shoot and root tissues after 6, 12 and 24 h of exposure to salt stress conditions, indicating the continuous effect of salinity on the growth of these cultivars (Table 7.3.3). The Skiff genotype shoots showed a slight increase in their Na^+/K^+ ratio index from 1428.35% to 1522.22% between 6 h to 12 h, however this reduced to 1069.75% after 24 h of salinity exposure. The Arivat genotype shoots remained almost unchanged in their Na^+/K^+ ratio index between 6 and 24 h of salt exposure, whereas the Na^+/K^+ ratio index in the roots increased continuously over the 24 h of salt stress exposure. This could indicate that the accumulation of $[Na^+]$ and $[K^+]$ in the root tissue may not impact on the ion accumulation in the shoots over a specific time or be due to a specific species. The Na^+/K^+ ratio index in the roots of the Calmariout and Hindmarsh genotypes remained almost unchanged between 6 and 12 h of salt exposure, but increased after 24 h of exposure, indicating that the salinity may have an effect on the root tissue after 12 h of exposure. Another difference was observed in the Buloke genotype shoots, where the Na^+/K^+ ratio index reduced to a greater degree after 12 h of salt exposure than it did after 6 and 24 h of treatment. This may be due to the presence of some response genes that could be more likely to reach a peak in their expression after 12 h, as suggested by Seki and colleagues in their study of *Arabidopsis* (Seki et al., 2002). This would require an in-depth study to allow a clear explanation to be obtained for this result. In general, accumulation of ions occur to a greater extent in the shoots than the root under salt stress conditions, as observed in the study performed by Ahmed et al. (2013).

Table 7.3.3 Na^+/K^+ ratio index based on salt tolerant barleys at different time-points

Variety	**Na^+/K^+ ratio**					
	Shoot			**Root**		
	6 hours	**12 hours**	**24 hours**	**6 hours**	**12 hours**	**24 hours**
Calmariout	1026.15	1175.00*	1337.30	250.46	247.56*	513.66
Mundah	659.74	1327.27*	1632.45	236.19	298.71*	479.25
Hindmarsh	606.183	1263.63*	1765.89	263.90	265.00*	571.12
Buloke	1733.84	1345.45*	1675.39	492.99	590.32*	623.54
Vlamingh	1332.07	1480.00*	1748.91	389.49	762.74*	773.73
Skiff	1428.35	1522.22*	1069.75	620.45	1043.90*	1148.85

CM72	1441.53	1600*	1847.65	792.15	1030.95*	1270.79
Arivat	1643.43	1633.3*3	1649.72	551.80	1087.50*	1342.13
Gairdner	995.14	1687.5*	2169.99	576.54	1070.73*	1546.80

*: data from Kamboj et al. (2015)

7.4 Evaluation of the salt tolerance of barley varieties

Barley is cultivated on a wide range of habitats, found scattered across the world, with 378 000 accessions being reported in the world's gene bank (van Hintum and Menting, 2003). During the process of evolutionary mutation, a wide genetic diversity of barley was created from a combination of domestication and breeding; this genetic diversity makes it reasonably straightforward to identify stress tolerant genotypes. Salinity is currently a global concern, predicted to expand (Wild, 2003). Hence, finding salt tolerant cultivars is a research focus worldwide. A strong correlation between barley habitats and levels of salt tolerance was been reported in Israel (Pakniyat et al., 1997). Salinity impedes the extent water uptake in plants, reducing the internal water content and changing the levels of hormone production (Atwell et al., 1999). Changing the internal water content is correlated to the tolerance level of cultivars as it shows the ability of cells to retain water (Khakwani et al., 2011). A study of wheat, soybean and barley showed that the ability of a plant to maintain its RWC in salt tolerant varieties was higher than that found in salt sensitive genotypes (Matin et al., 1987). In addition, salt tolerant species were reported to possess a Na^+ exclusion mechanism (Greenway and Munns, 1980). Among the various crops species, barley is a strong-ion exclusion plant, with the ability to maintain a low internal ion concentration against a high ion concentration in the soil in which it is cultivated (Atwell et al., 1999). Barley also shows a high efflux of K^+ that may restrict the accumulation of Na^+ in the shoots (Chen et al., 2007). The ability to maintain a low Na^+/K^+ ratio has been used as key feature for evaluation of the salt tolerance in barley (Chen et al., 2007, Chen et al., 2005). Therefore, the ability to maintain high RWCI and low Na^+/K^+ index were used as criteria for the identification of salt tolerance (Ahmed et al., 2013, Witzel et al., 2014, Chen et al., 2005).

As seen in Table 7.4.1, the results highlighted the good correlation between the RWC and Na^+/K^+ index in some tested genotypes, however, this correlation was not seen in other cultivars in response to salt stress. Similar results were also found between the relative tissue biomass and ion ratios in barley (Qiu et al., 2011) and bread wheat (Genc

et al., 2007). Plants were reported to respond to salt stress based on their naturally stomatal behaviour on a time scale ranging from minutes to hours (Munns et al., 2010). In addition, potassium essentiality has been proven as a key role in the major inorganic osmoticum, impacting the extent of stomatal opening and closing that occurs (Marschner, 1995), resulting in an indirect effect on the RWC. The Na^+/K^+ index in the shoots was usually used to determine the salt tolerance, as it provided an indication regarding the plant's ability to avoid ion toxicity (Munns et al., 1995). Recently, the ability to retain K^+ in roots has been shown to be another important criterion for salt tolerance in barley and wheat (Cuin et al., 2008, Chen et al., 2007). Hence, the Na^+/K^+ index in roots and shoots measured over a 24 h period of salt exposure was used as a critical criterion in the evaluation of salt tolerance in the present study. Tolerance rankings were then assigned to the genotypes based on the loss in RWC and Na^+/K^+ index in the roots and shoots of the cultivars tested, between 6 and 24 h of salt exposure. 15 barley varieties were able to be divided into three groups: a salt tolerant group (Calmariout and Stirling genotypes), a salt-moderated group (Arivat, Buloke, Dask, Hindmarsh, Kaputar, Lofty Nijo, Mundah, Skiff and Steptoe genotypes) and a salt-sensitive group (CM72, Gairdner, Morex and Vlamingh genotypes) (Table 7.4.1).

Identifying the Gairdner genotype as being the salt sensitive cultivars is consistent with the work reported by Chen et al. (2005). The Morex and CM72 genotypes have, however, been classified as being salt tolerant in other studies (Chen et al., 2005, Witzel et al., 2014, Ahmed et al., 2013), whereas they were found to be salt-sensitive in the present study. The difference in these results may be due to the way in which the salt exposure was applied. In this study, the Morex and CM72 genotypes were exposed to acute stress conditions (150 mM NaCl for 6 and 24 h) whereas the Morex genotype was subjected to chronic stress conditions (150 mM NaCl for 10 days) in the study performed by Witzel et al. (2014) and the CM72 genotype was treated with 100 mM NaCl for 8 days (Ahmed et al., 2013), which is less acute than the conditions used in this study. Among the tested cultivars, the Calmariout genotype has been proposed as being salt tolerant, which is in agreement with the findings reported by Kamboj et al. (2015). This study found that the Arivat and Hindmarsh genotypes, however, were classified as being salt moderated whereas Kamboj et al. (2015) classified these genotypes as being salt sensitive and tolerant, respectively. These discrepancies may be due to the time over which the plants were exposed to the salt conditions. These cultivars did not fully recover to a

reduced steady rate of growth after 12 h of salt treatment. The study performed by Termaat et al. (1985) showed that exposure to salinity had no further effect on the growth of barley over a period up to 8 days, suggesting that longer-term studies at the physiological level may allow better comparisons to be made. Among the tested genotypes, Stirling was classified as salt-tolerant, Skiff and Dask as salt moderate. The Stirling genotype is only grown in Western Australia, the most salt affected agricultural land in Australia, while the Skiff genotype is only grown in Queensland, the least salt affected region. The Dask genotype was cultivated in both areas (Victorian Winter Crop Summary, 2013). These results should be taken into account when considering the suitable cultivars for different regions.

Table 7.4.1 Salt tolerance indices of barley varieties based on physiological traits

Barley variety	Plant parameters and stress ranking at 6h salt stress				Plant parameters and stress ranking at 24h salt stress				Trend
	RWCI	Na^+/K^+ Index Average	RWCI rank	Na^+/K^+ index rank	RWCI	Na^+/K^+ Index Average	RWCI rank	Na^+/K^+ index rank	
Arivat	96.72	1097.62	**5**	**13**	96.42	1495.92	**7**	**13**	**Steady moderate**
Buloke	87.95	1113.41	**11**	**14**	103.70	1149.46	**2**	**8**	**Improving moderate**
Calmariout	100.35	638.30	**3**	**4**	96.58	925.48	**6**	**1**	**Improving tolerant**
CM72	94.69	1116.84	**7**	**15**	92.58	1559.22	**9**	**14**	**Steady sensitive**
Dask	71.21	820.10	**15**	**8**	99.11	1323.13	**4**	**11**	**Improving moderate**
Gairdner	91.56	785.84	**9**	**7**	76.42	1858.40	**14**	**15**	**Declining sensitive**
Hindmarsh	86.90	435.04	**12**	**1**	98.28	1168.50	**5**	**9**	**Steady moderate**
Kaputar	92.14	890.89	**8**	**10**	89.45	1115.56	**13**	**6**	**Steady moderate**
Lofty Nijo	95.12	1021.69	**6**	**11**	95.24	943.69	**8**	**2**	**Improving moderate**
Mundah	104.79	447.96	**1**	**2**	91.73	1055.85	**10**	**4**	**Declining moderate**
Morex	86.29	636.08	**13**	**3**	70.57	1138.97	**15**	**7**	**Declining sensitive**
Skiff	100.68	1024.40	**2**	**12**	91.12	1109.30	**11**	**5**	**Steady moderate**
Steptoe	73.11	735.81	**14**	**5**	100.33	1380.60	**3**	**12**	**Steady moderate**
Stirling	90.80	742.38	**10**	**6**	103.92	987.77	**1**	**3**	**Improving tolerant**
Vlamingh	98.44	860.78	**4**	**9**	90.44	1261.32	**12**	**10**	**Declining sensitive**

7.5 Summary

In this study, 15 barley varieties were studied for their tolerance to salt stress conditions (150 mM NaCl for 6 h and 24 h). The RWC index and Na^+/K^+ ratio index were used as key features for the evaluation of the barley tolerance to salt conditions, and a diverse response of these tested cultivars between 6 and 24 h treatment was observed. Correlation between the RWCI and Na^+/K^+ ratio index results was not observed for all genotypes tested, since the salt tolerance evaluation based on the Na^+/K^+ ratio index is not consistent with that associated with the RWCI. The trend in the Na^+/K^+ ratio index in shoots and roots over 24 h of salt exposure were chosen as critical criteria in considering the important role plays by K^+ in plant growth and stomatal opening and closing, in addition to the toxicity of Na^+ accumulation. Hence, based on the changes of RWCI and Na^+/K^+ ratio index over a 24 h period of exposure to 150 mM NaCl, the Calmariout and Stirling varieties were classified as being salt-tolerant, whereas the CM72, Gairdner, Morex and Vlamingh varieties were classified as being salt sensitive. These varieties would, therefore, make good candidates for further studies on gene-related salt tolerance.

Chapter 8.

Gene expression analysis of some abiotic responsive genes in barley

8.1 Overview

Soil salinity has been proven to impede the growth of plants and their yield by causing osmotic and ion-specific stress, which currently affects 5.7 million ha of agricultural land in Australia. It is predicted that another 17 million ha of land will be affected by salinization by 2050 (as discussed in Chapter 2). In addition to the existing evidence of the roles of miRNAs to environmental stresses, a complicated genetic network has been reported in the literature to regulate gene expression in response to the detrimental effects of abiotic stress (as detailed in Chapter 2). The preceding chapter 6 presented a comprehensive analysis of barley miRNAs and their potential roles in response to salinity. The targets of these miRNAs were also predicted; the functional roles of these targets are, however, less understood. In this study, expression pattern of some miRNA targets such as squamosa promoter-binding protein (SPL), GRAS transcription factor (SCL) and MYB6 were compared to that of other reported stress responsive genes, choline monooxygenase (CMO), betaine aldehyde dehydrogenase (BADH) and ABA insensitive gene (ABI), in response to salinity in the leaf and root tissues of some of the salt-resistant Australia barley varieties identified in Chapter 7. The research would provide basic information on the role of tested miRNA targets as well as screening of candidate genes that could be employed for developing salt-tolerant crops. These genes are discussed in detail below:

MYB is one of the largest functional transcription factor (TF) families in plants that contain the common DNA-binding domain (MYB domain) (Dubos et al., 2010). The transcript encoding MYB was identified as the target of hvu-miR159 (Dryanova et al., 2008). Depending on the number and repeating units of the MYB domain, MYB has been classified into four groups: 4R-MYB, R1R2R3-MYB, R2R3-MYB and 1R-MYB. Among these, R2R3-MYB is the largest and most commonly found group in plants (Du et al., 2012a). The MYB family has been found to be involved in many plant-specific processes, such as developmental processes, primary and secondary metabolism, cell fate and identity (Dubos et al., 2010, Katiyar et al., 2012) or in response to various biotic and abiotic stresses (Li et al., 2014). CMO and BADH are important enzymes that are involved in the synthesis of glycine betaine (GB) in plants, which takes place in two steps. In the first, the CMO converts choline to betaine aldehyde; then in the second step, the BADH catalyzes betaine aldehyde to form GB (Nakamura et al., 1997). GB is a

compatible solute that acts as an osmo-protectant for maintaining the osmotic balance (Robinson and Jones, 1986) and as a chemical chaperone for protecting the enzyme activity and membranes against the inhibitory effects of salt and temperature stress (Incharoensakdi et al., 1986, Murata et al., 1992). The basic leucine zipper (bZIP) transcription factor family has an important role in developmental and physiological plant processes such as photo-morphogenesis, leaf and seed formation, stress response and signal transduction (Uno et al., 2000). SCARECROW-LIKE (SCL) genes are novel genes that belong to the GRAS family, which have a diverse role in the growth and development of plants (Bolle, 2004), while SQUAMOSA-PROMOTER BINDING PROTEIN LIKE (SPL) proteins are a family of transcription factors that have diverse funtional roles in flowering time (Xie et al., 2006), branching (Miura et al., 2010), shoot maturation (Schwarz et al., 2008), pollen sac development (Unte et al., 2003) or copper homeostasis (Yamasaki et al., 2009). The transcripts encoding SCL and SPL proteins are the targets of hvu-miR171 and hvu-miR156, respectively (Dryanova et al., 2008).

8.2 Quality of cDNA synthesis

After DNase treatment, the total RNA obtained from leaf and root samples of both the control and salt-stressed plants was reverse transcribed into cDNA. Each cDNA preparation was tested for quality (lack of gDNA contamination) by amplification using intron-flanking actin primers. The primers of the actin gene were designed to span introns, using the Unigene sequences as a template, according to the following criteria: minimal secondary structures, comparable annealing temperatures between primer pairs and GC content of approximately 50% was used. The cDNA was used for further analysis if the PCR showed a single band at approximately 147 bp in the gel image (Figure 8.2.1).

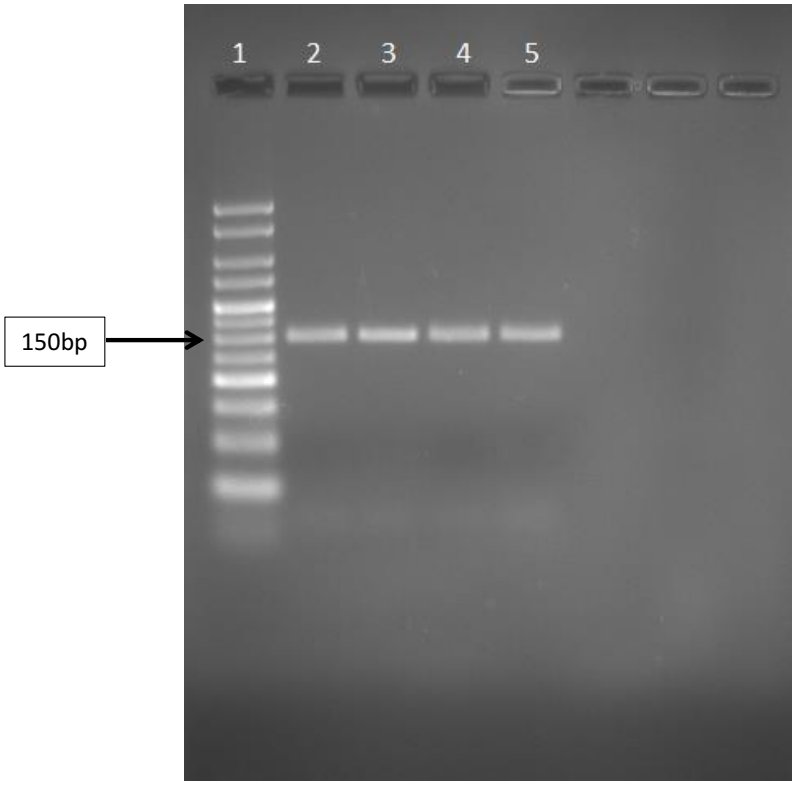

Figure 8.2.1 Example of a gel image showing the quality of the cDNA.

Lane 1: GenerulerTM 50bp DNA ladder, Lanes 2-5: Actin (AK252278) PCR products from different cDNA preparations.

8.3 Specificity of primers

Six genes, i.e., SPL, MYB6, SCL, CMO1, BADH1 and ABI5 were selected according to their abiotic stress responsive ability as well as their functional role in the growth and development of plants. This allowed their differential expression under salt stress to be studied. Thus, the differential expression of these genes was investigated in response to the presence of salinity growth conditions. The gene expressions were determined from both leaf and root samples to see whether the gene expression was tissue-specific, and to study their role in different parts of the plant.

To investigate the differential expression of selected genes, eight barley plants (*Hordeum vulgare* cv. Arivat, Buloke, Calmariout, CM72, Gairdner, Hindmarsh, Steptoe and Stirling) were subjected to salt stress growth conditions (150 mM NaCl for 24 hours, 14 days growth). The total RNA of these plants was isolated from the leaf and root tissues

in triplicate for each control and salt-stressed group. The cDNA was synthesized and tested as previously described. The qualified cDNA was then used for determining gene expression levels by real-time PCR using the SensiFAST™ SYBR & Fluorescein Kit (Bioline). All the primers for the tested genes were designed using the UniGene sequences in the National Center for Biotechnology Information (NCBI http://www.ncbi.nlm.nih.gov/) as templates. The expression of barley genes was normalized using a house-keeping gene (α-tubulin) as an internal control. The specificity of each primer pair was tested and an optimized annealing temperature was determined using gradient PCR to obtain the clear bright products present on an agarose gel. The specificity of these primers was confirmed by visualizing single bands which showed the expected sizes presented in Table 3.8.1 compared to the marker on the gel (Figure 8.3.1).

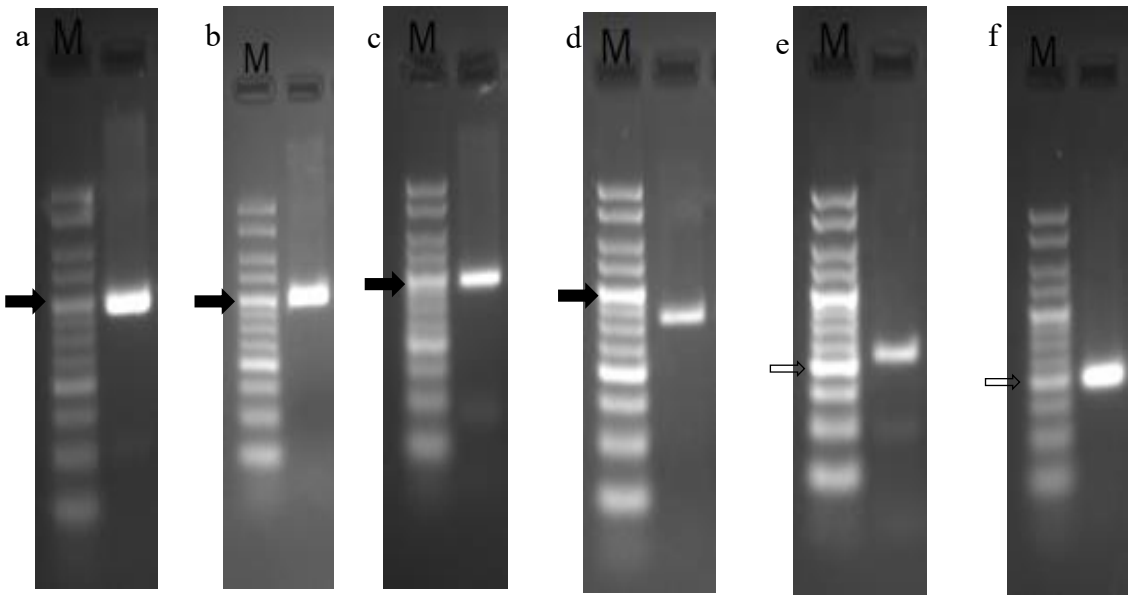

Figure 8.3.1 Gel image showing the amplified PCR products.

The markers are denoted as M (Hyperladder V); the amplicons amplified using the following primers **a**. HvABI5F/R; **b**. HvMYB6F/R; **c**. HvSCLF/R; **d**. HvSPLF/R; **e**. HvCMO1F/R; **f**. HvBADH1F/R. The relevant marker bands are shown as 100 bp (➡) and 200 bp (⟹).

8.4 Modulation of MYB6 expression under salinity growth conditions

The MYB transcription factors were identified for a number of diverse species. For example, 244 MYB transcription factors were found in soybean (Du et al., 2012b), 192 in *Populus trichocarpa* (Wilkins et al., 2009), 126 in *Arabidopsis thaliana* (Stracke et al., 2001, Dubos et al., 2010, Yanhui et al., 2006), 108 in *Vitis vinifera* (Matus et al., 2008), 157 in *Zea mays* (Du et al., 2012a), 88 in rice (Katiyar et al., 2012) and 22 in wheat (Zhang et al., 2012). MYB transcription factors have been proven to regulate abiotic stress tolerance in plants. Many MYB genes identified in rice and wheat (OsMYB2, OsMYB3R-2, OsMYB4, OsMYBS3, TaMYB2, TaMYB32, TaMYB56, TaMYB30 and TaMYB73) have been characterized as important component in abiotic stress response pathway (Ma et al., 2009, Mao et al., 2011, Vannini et al., 2004). A wheat MYB gene, TaMYB19, has been shown to regulate the expression of a number of abiotic stress-related genes, leading to enhance abiotic tolerance in *Arabidopsis* (Zhang et al., 2014a). Overexpression of MYB have been reported in plants to enhanced tolerance under different abiotic stress conditions, such as drought (Oh et al., 2011), cold (Zhou et al., 2015), ABA and salt stress in *Arabidopsis* (Kim et al., 2015), salt, cold and dehydration tolerance in rice (Yang et al., 2012) or drought, salt and cold stress in wheat (Cai et al., 2011). There is, however, still few studies reported pertaining to the MYB values for barley. The first report of barley MYB was provided by Tombuloglu et al. (2013), who identified 51 R2R3-MYB genes from leaf and root tissues. It was also found that these genes played a role in response to boron stress conditions.

For the conditions used in the current study, Hindmarsh and Arivat showed no noticeable change in the expression of MYB6 in both leaf (FC, 1.12 ± 0.71 and 1.15 ± 0.57) and root (FC, -1.02 ± 0.23 and -1.42 ± 0.47) tissues, respectively. The Buloke barley exhibited a significant increase in expression of MYB6 in the leaf tissue (FC, 39.20 ± 1.42) while the expression of MYB6 decreased in the root tissue (FC, -2.28 ± 1.77). Expression of MYB6 was up-regulated in both the leaf and root tissue of CM72 barley (FC leaf, 5.48 ± 0.26 and FC root, 1.74 ± 1.20) and Gairdner barley (FC leaf, 1.51 ± 0.49 and FC root, 6.23 ± 1.04). Calmariout barley showed up-regulation of MYB6 in the leaf tissue (FC, 3.70 ± 0.73) and down-regulation in the root tissue (FC, -1.29 ± 0.47). Conversely, the Stirling barley displayed a decrease in MYB6 in the leaf tissue (FC, -3.33 ± 0.63) and an increase in the root tissue (FC, 2.10 ± 0.92), while MYB6 expression was

found to be reduced in both the leaf and root tissue of the Steptoe barley (FC -8.00 ± 0.79 and -5.18 ± 1.10, respectively) (Table 8.4.1; Figure 8.4.1).

Table 8.4.1 Fold change in expression of selected barley genes in response to salt stress growth conditions (150 mM NaCl, 24 h).

Barley		**Gene Fold change**					
		MYB6	**BADH**	**CMO**	**ABI5**	**SCL**	**SPL**
Buloke	Leaf	39.2 ± 1.42	1.27 ± 0.34	1.35 ± 0.78	2.12 ± 0.08	1.88 ± 0.21	2.52 ± 0.24
	Root	-2.28 ± 1.77	2.02 ± 0.27	4.12 ± 0.45	-1.69 ± 1.04	2.76 ± 0.71	2.59 ± 0.20
Calmariout	Leaf	3.70 ± 0.73	-2.42 ± 1.81	1.26 ± 0.74	4.68 ± 0.25	2.11 ± 0.56	6.11 ± 0.70
	Root	-1.29 ± 0.47	2.51 ± 1.22	-9.52 ± 0.87	2.21 ± 1.10	-25.64 ± 0.93	-19.23 ± 0.30
Stirling	Leaf	-3.33 ± 0.63	-1.85 ± 0.53	-1.36 ± 0.80	-2.45 ± 0.63	1.06 ± 0.78	6.58 ± 0.19
	Root	2.10 ± 0.92	1.37 ± 0.61	1.65 ± 0.63	1.90 ± 0.69	4.82 ± 1.51	2.13 ± 0.99
Arivat	Leaf	1.15 ± 0.57	1.06 ± 0.34	1.23 ± 0.37	2.21 ± 0.35	-1.18 ± 0.32	1.20 ± 0.78
	Root	-1.42 ± 0.47	-1.36 ± 0.19	-1.15 ± 0.17	2.03 ± 0.15	1.87 ± 0.35	-1.04 ± 0.18
CM72	Leaf	5.48 ± 0.26	7.85 ± 0.45	3.53 ± 1.44	1.37 ± 0.31	2.51 ± 1.03	8.95 ± 0.88
	Root	1.74 ± 1.20	-1.17 ± 0.49	6.07 ± 1.03	-1.85 ± 0.42	1.02 ± 0.14	1.72 ± 0.70
Steptoe	Leaf	-8.00 ± 0.79	-4.03 ± 0.31	-40.00 ± 1.57	-4.71 ± 1.51	-28.57 ± 1.27	-1.36 ± 1.27
	Root	-5.18 ± 1.10	-3.22 ± 0.81	-5.81 ± 0.84	-3.62 ± 0.07	-3.21 ± 0.39	-2.65 ± 0.40

Barley		**Gene Fold change**					
		MYB6	**BADH**	**CMO**	**ABI5**	**SCL**	**SPL**
Hindmarsh	Leaf	1.12 ± 0.71	1.66 ± 0.36	5.85 ± 0.32	1.98 ± 0.72	3.15 ± 0.57	1.77 ± 0.42
	Root	-1.02 ± 0.23	1.28 ± 0.25	-1.72 ± 0.11	-1.26 ± 0.81	-1.17 ± 1.02	1.75 ± 0.35
Gairdner	Leaf	1.51 ± 0.49	-8.19 ± 0.32	-13.15 ± 0.62	-1.50 ± 0.75	5.09 ± 0.73	14.4 ± 0.71
	Root	6.23 ± 1.04	1.72 ± 0.10	2.55 ± 0.18	3.85 ± 0.58	3.98 ± 1.34	1.28 ± 0.21

MYB expression has been proven to influence the amount of anthocyanin pigment being synthesized in tomato plants (Wada et al., 2014) and positively respond to water deficiency conditions (Koops et al., 2011). A recent study also found that the R2R3-MYB barley type controls the amount of anthocyanin pigment being formed in different vegetative tissues (Himi and Taketa, 2015). Nakabayashi et al. (2014) found that the overexpression of MYB will prevent water loss, one important factor in the ability to withstand the negative effects of salt growth conditions. Overexpression of MYB also conferred improved salt tolerance in *Arabidopsis* (Cheng et al., 2013). Expression of MYB was found to be induced after 24 hours of salt treatment, with a resulting of > 20 fold increase in rice (Zhu et al., 2015) and > 3 fold increase in wheat (Cai et al., 2011). These results highlighted the positive growth role associated with MYB expression under salt stress conditions. In the current study, MYB6 expression increased in most tested varieties, particularly in the leaf tissue of Buloke barley where a greater than 39 fold-increase was observed, indicating the positive role of this gene in barley in response to growth under salt stress conditions. MYB6 expression was much lower in the root tissues compared to that of the leaves, suggesting that MYB6 expression might not play as a great role in the root tissue. This suggestion is in agreement with the data reported by Munns and Termaat (1986), who reported that leaf growth was more sensitive to salinity and nutrient stress conditions than root growth. The significant decrease in the expression of MYB6 in the leaf and root tissue of Steptoe barley in the current study is inconsistent with the growth results obtained, as reported in Chapter 7. In addition, the Gairdner barley was classified as being salt sensitive in the current study (Chapter 7), whereas an increase in MYB6 expression was observed in both the leaf and root tissues, particularly in the root tissue with a greater than 6-fold increase. The results suggest tissue-specific or cultivar-specific expression of MYB6. MYB6 expression has also been reported to play a role in initiating disease resistance signaling (Chang et al., 2013), but not to boron stress conditions in the growth of barley (Tombuloglu et al., 2013), indicating the specific response of MYB6 to environmental stress. In addition, expression of MYB6 was reported to trigger anthocynanin accumulation in *Chrysanthemum morifolium ramat* (Xiang et al., 2015) and *Asiatic hybric lily* (Yamagishi et al., 2010) that confer tolerance to abiotic stress (Gould, 2004). Therefore, overexpression of MYB6 might be involved in conferring salt tolerance in barley.

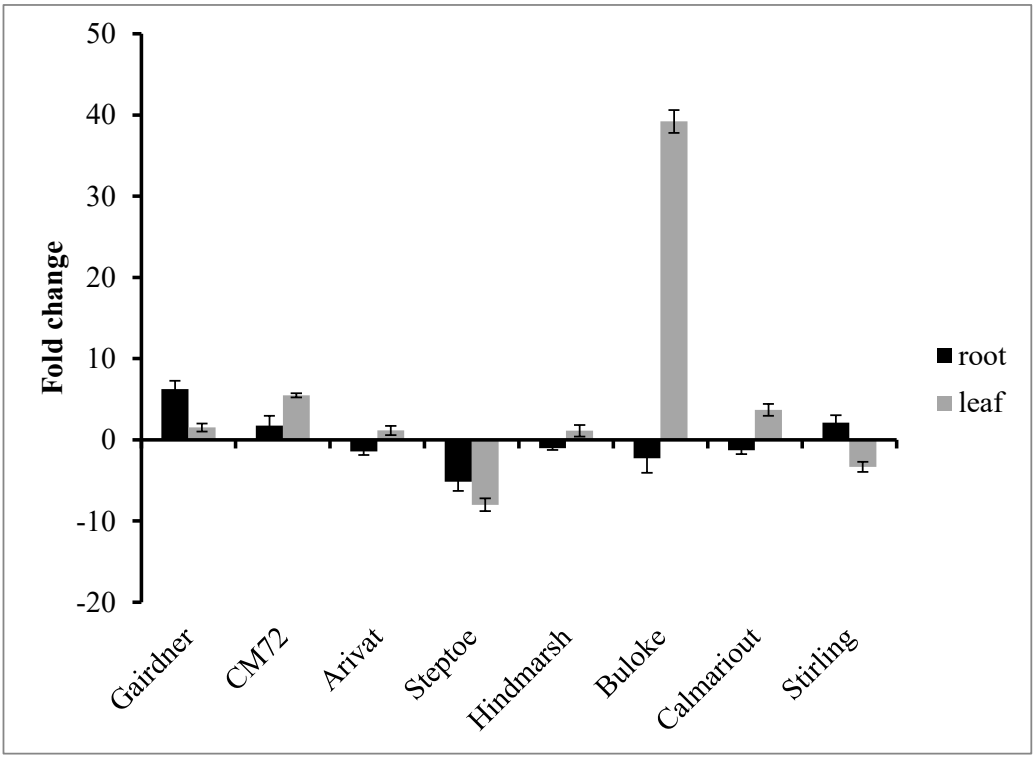

Figure 8.4.1 Differential expression of MYB6 in the leaf and root tissues of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

8.5 Modulation of BADH1 and CMO1 expression under salinity

CMO and BADH have been reported to have an important role in the synthesis of glycine betaine (GB), an osmoprotectant for maintaining the osmotic balance (Robinson and Jones, 1986) and protection of the enzyme activity and the membranes of plants under abiotic stress (Incharoensakdi et al., 1986, Murata et al., 1992). Accumulation of GB in the roots or leaves of rice has been shown to enhance its salt tolerance (Harinasut et al., 1996).

After 24 hours treatment with 150 mM NaCl, the expression of CMO1 was almost unchanged in the leaf tissues of barley cultivars, with the exception of increases in CM72 (FC, 3.53 ± 1.44) and Hindmarsh (FC, 5.85 ± 0.32) barleys, and dramatic decreases in Gairdner (FC, -13.15 ± 0.62) and Steptoe (FC, -40.00 ± 1.57) cultivars. In contrast, CMO1 expression was differerent in the roots of all tested samples, increasing in the Buloke, CM72, Gairdner and Stirling barleys and decreasing in the Arivat, Calmariout, Hindmarsh and Steptoe barleys. The highest expression level was

found for CM72 barley (FC, 6.07 ± 1.03), followed by Buloke barley (FC, 4.12±0.45). The lowest CMO1 expression was found for the Calmariout (FC, -9.52 ± 0.87) and Steptoe (FC, -5.81 ± 0.84) barleys (Table 8.4.1; Figure 8.5.1).

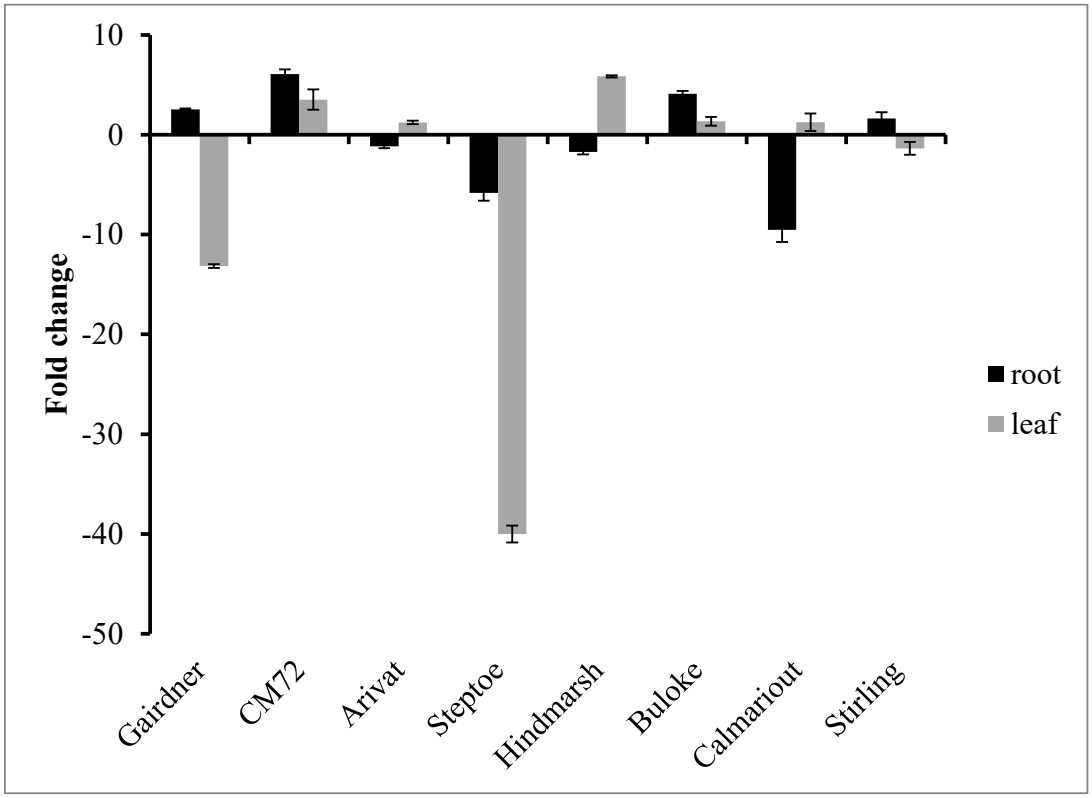

Figure 8.5.1 Differential expression of CMO1 in the leaf and root tissues of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

The second enzyme in the GB synthesis pathway, BADH1, showed a different expression pattern. In leaf tissue, BADH1 was down-regulated in the Gairdner (FC, -8.19 ± 0.32), Steptoe (FC, -4.03 ± 0.31), Calmariout (FC, -2.42 ± 1.81) and Stirling (FC, -1.85 ± 0.53). BADH1 expression increased in the CM72 (FC, 7.85 ± 0.45) and Hindmarsh (FC, 1.66 ± 0.36) barleys, but little or no change was observed for the Buloke (FC, 1.27 ± 0.34) and Arivat (1.06 ± 0.34) barleys. In the root tissue samples, BADH1 was up-regulated in almost all cultivars, except Steptoe (FC, -3.22 ± 0.81) barley. The highest level of BADH1 expression was observed in the Calmariout (FC, 2.51 ± 1.22) barley root tissues (Table 8.4.1; Figure 8.5.2).

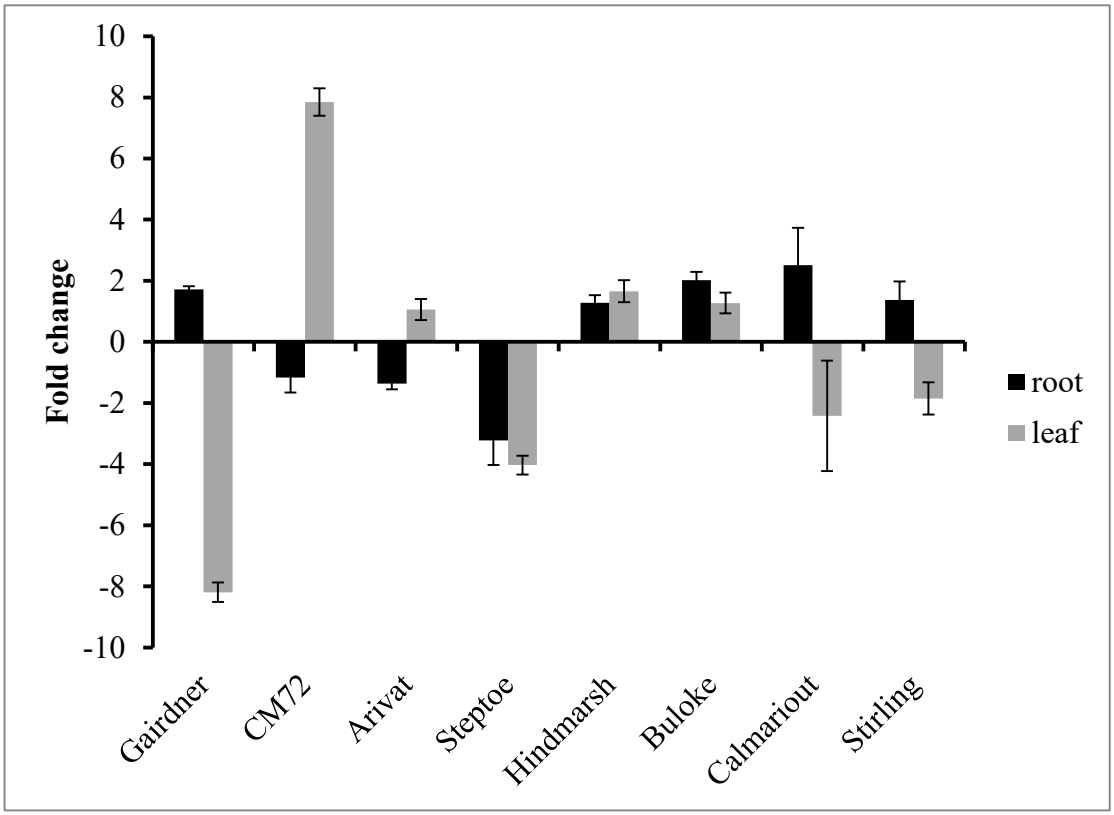

Figure 8.5.2 Differential expression of BADH in the leaf and root tissues of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ±SE (n=3).

CMO1 and BADH1 are involved in the GB synthesis pathway. Thus, it is advantageous to observe the changes in CMO1 and BADH1 expression under stress conditions. As shown in Figure 8.5.3, CMO1 and BADH1 showed the same direction of expression in the leaf tissue of all barley varieties, except Calmariout barley, where an increase in CMO1 and decrease in BADH1 expression was observed. As expected, CMO1 and BADH1 expression significantly decreased in the Gairdner barley, classified in this study as being salt sensitive. CM72 barley, however classified here as a salt sensitive cultivar, showed an increase in the expression of two genes, particularly BADH1. It has also been found that an increase in the GB level correlated to an increased BADH expression in barley (Arakawa et al., 1990). BADH expression has been reported to be responsive to salt, drought and temperature stresses (Fitzgerald et al., 2009). Therefore, on the basis of this information, proposing CM72 as being a salt tolerant cultivar is inconsistent with the findings of the current study and that of Kamboj et al. (2015), but consistent with that reported in the study by Ahmed et al. (2013). Similarly, Steptoe barley showing significant decrease in the expression of

both CMO1 and BADH1 did not support the classification obtained in Chapter 7; however, support the report of being salt sensitive in the study by Witzel et al. (2009). Calmariout and Stirling barleys, being classified as salt tolerant, exhibited almost no change in either CMO1 and BADH1 expression. Another study also found no change in CMO expression in the leaf tissues of Haruna-nijyo barley after 24 h of exposure to salt treatment (Mitsuya et al., 2011). The results may suppose the cultivar-specific expression of CMO and BADH among genotypes.

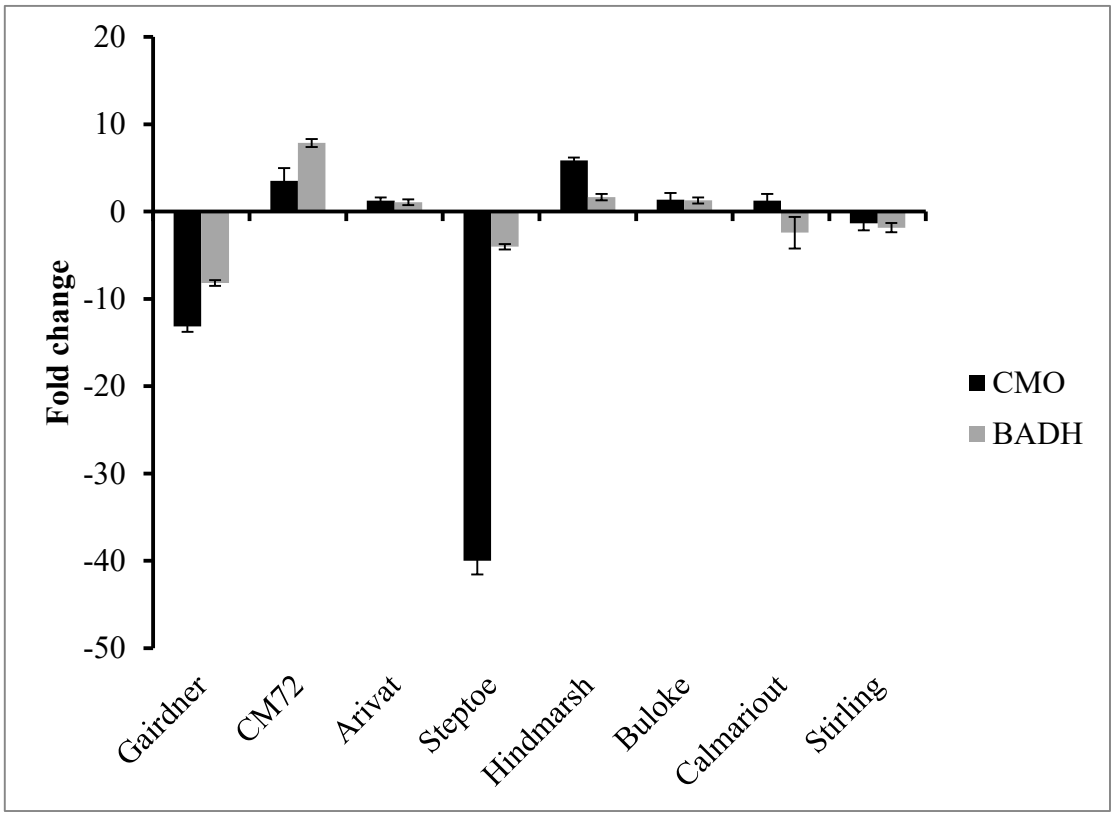

Figure 8.5.3 Differential expression of CMO and BADH in the leaf tissue of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

In root tissue, the two genes were found to have increased in Buloke, Stirling and Gairdner barleys, but decreased in Arivat and Steptoe barleys, while showing the opposite direction of expression in Calmariout, CM72 and Hindmarsh barleys (Figure 8.5.4). BADH1 expression increased in the leaf and root tissues of barley under salt stress, with the expression being much higher in the leaf than root tissue (Ishitani et al., 1995, Arakawa et al., 1990). The variance in BADH expression could be explained by the difference in cultivars and the conditions used in the Arakawa et al. study (cv. Haruna Nijyo at 200 mM NaCl for 2 days) and the Ishitani et al. study (cv. 116 Jeonju

Native Korean at 300 mM NaCl for 2 days). CMO is known as being the initial enzyme taking part in the glycine betaine synthesis pathway. The activity of BADH is thought to be dependent on the extent of CMO accumulation that has taken place during GB synthesis under salinity conditions. BADH is an important enzyme that plays a key role in osmotic adjustment (Zhang et al., 2015a). In addition, studies on rice and barley have shown that BADH was not only involved in GB production, but was also produced in response to abiotic stress conditions (Nakamura et al., 1997, Nakamura et al., 2001). BADH1 may catalyze the production of GABA from GA-Bald (Fujiwara et al., 2008), which is an osmoprotectant (Mazzucotelli et al., 2006). Therefore, overexpression of BADH has the ability to confer salt tolerance in barley. When considering of the expression of these two genes in both leaf and root tissues, it is largely agreed that the classification of barleys as being salt resistant should be based on a physiological test (as reported in Chapter 7). Here, the Gairdner barley was classified as being salt sensitive, the Arivat barley as salt moderated and Buloke barley as being more salt tolerant.

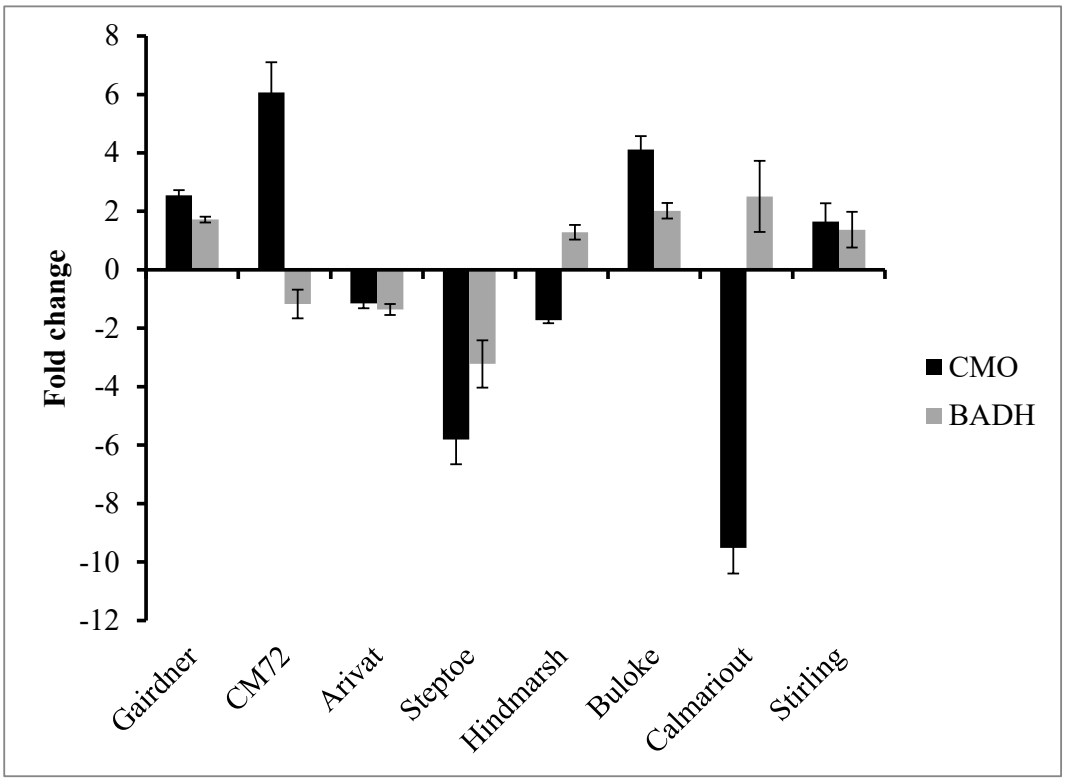

Figure 8.5.4 Differential expression of CMO and BADH in the root tissue of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

5.6 Modulation of ABI5 expression under salinity conditions

ABI5, a member of the bZIP transcription factors, is responsible for the ABA induction of gene expression (Casaretto and Ho, 2003). ABI5 was found to regulate the expression of ABRE-dependent genes that enhanced the extent of osmotic adjustment (Finkelstein et al., 2005), seed dormancy and seedling growth (Miura et al., 2009). Zou et al. (2008) have also demonstrated that the overexpression of OsABI5 enhanced the extent of salt tolerance in rice. An ABI-like transcription factor gene (TaABL1), having a high protein sequence similarity to barley ABI5, has been reported to be produced as a result of high salt stress conditions. Its overexpression improved the salt stress tolerance by regulating the downstream genes that controlled the degree of stomatal closure (Xu et al., 2014). Hence, ABI5 expression was selected as the method for detecting tolerance to salt stress in barley cultivars.

The expression of HvABI5 showed a clear direction among the barley groups being investigated. In leaf tissue, the HvABI5 was found to increase in the salt moderated group (Hindmarsh, Buloke and Arivat barleys) and salt tolerant Calmariout barley, with the exception of the Steptoe and Stirling barleys, which showed a reduction in HvABI5 expression. In the salt sensitive group, the Gairdner barley exhibited a decrease in the HvABI5 expression, but the CM72 barley showed almost no change of this gene. The highest level of HvABI5 expression was found in the Calmariout barley (FC, 4.68 ± 0.25), followed by the Arivat (FC, 2.21 ± 0.35) and Buloke (FC, 2.12 ± 0.08) barleys. The lowest level of HvABI5 expression was found in the Steptoe (FC, -4.71 ± 1.51) barley, followed by Stirling (FC, -2.45 ± 0.63) and Gairdner (FC, -1.50 ± 0.75) barleys. ABI5 expression was diverse in the root tissue of all tested cultivars, showing an up-regulation in Gairdner (FC, 3.85 ± 0.58), Calmariout (FC, 2.21 ± 1.10), Arivat (FC, 2.03 ± 0.15) and Stirling (FC, 1.90 ± 0.69) barleys and down-regulation in Steptoe (FC, -3.62 ± 0.07), CM72 (FC, -1.85 ± 0.42), Buloke (FC, -1.69 ± 1.04) and Hindmarsh (FC, -1.26 ± 0.81) barleys (Table 8.4.1; Figure 8.6.1).

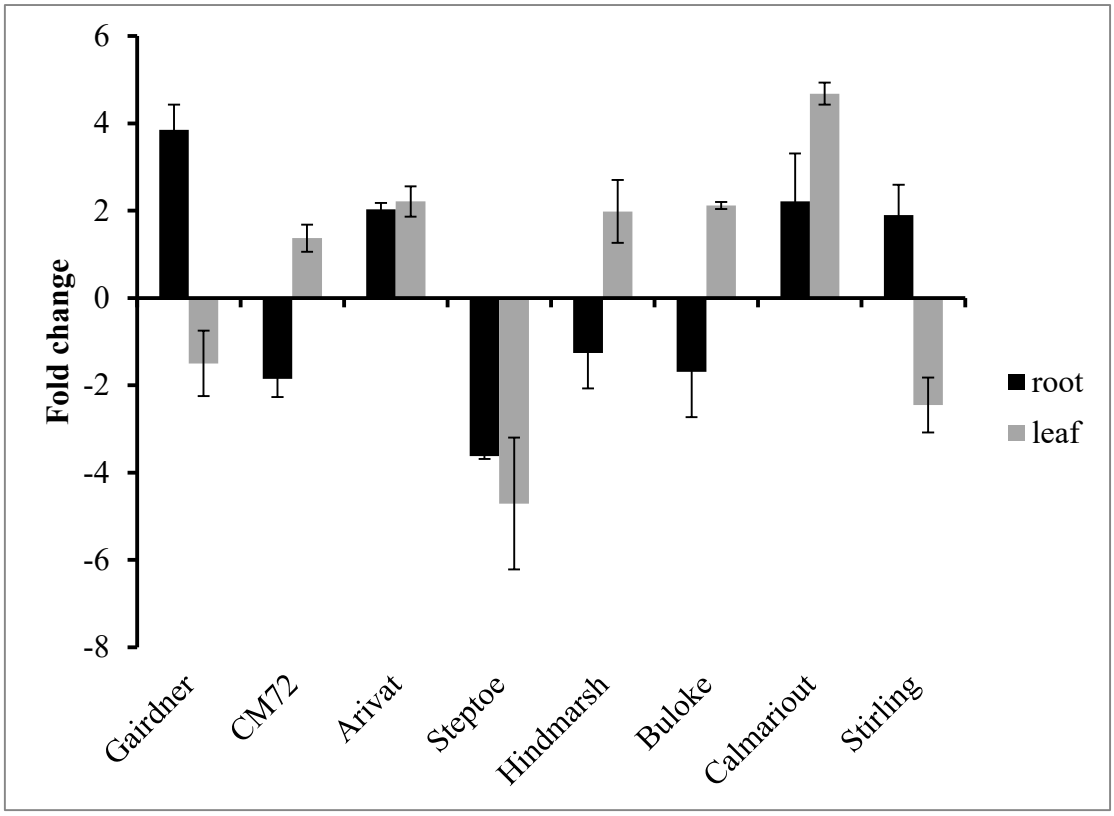

Figure 8.6.1 Differential expression of ABI5 in the leaf and root tissues of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

In general, ABI5 expression did not show significant differences in all samples compared to the other genes being investigated. This result was in agreement with the results reported by Brocard et al., who found ABI5 expression was strongly induced under ABA but very weakly induced under other stress treatment conditions (Brocard et al., 2002). Kobayashi et al. (2008) reported that Wabi5, a wheat HvABI5 ortholog, exhibited different expression patterns between two wheat cultivars possessing distinct levels of stress tolerance. The tested barley varieties can, therefore, be classified according to their expression of ABI5 in both leaf and root tissues. As can be seen from the data presented in Figure 8.6.1, Calmariout barley showed up-regulation of ABI5 in both leaf and root tissue, classified as salt tolerant. The Calmariout and Arivat barleys exhibited the same direction of gene expression, with a higher expression in the Calmariout barley compared to the Arivat barley, indicating that the Arivat barley is more sensitive to salt exposure than the Calmariout variety. The Buloke, CM72 and Hindmarsh baleys, exhibiting similar expression patterns,

should be classified as being salt moderated varieties because the leaf tissue appears to be more sensitive to salt stress than the root tissue. The Gairdner and Stirling barleys exhibited the same pattern of ABI5 expression in both leaf and root tissues, and therefore are categorised as being salt sensitive while Steptoe barley exhibited down-regulation of ABI5 in both leaf and root tissue, being the sensitive variety. However, the study of Yan et al. (2012) reported that the overexpression of maize ABI5 resulted in a lowered activity of the superoxide dismutase and peroxidase content of proline, indicating a negative regulation on the salt stress response. A study of the targets of ABI5 expression showed that the transcription factor induced the production of genes involved in many processes such as seed maturation, metabolism, cell structure, stress response as well as the negative regulators of ABA signalling (Reeves et al., 2011). Therfore, ABI5 expression can be both positive and negative feedback in gene regulation responses to stress. Interestingly, MYB was found to negatively regulate the expression of ABI5 in *Arabidopsis* (Kim et al., 2015). Therefore, a more comprehensive study on the expression pattern of both the control and target genes of ABI5 is required to allow a further understanding of its function and allow intervarital comparisons to be made.

8.7 Modulation of SCL expression under salinity conditions

SCL expression is in its early stages of analysis, with 33 and 57 GRAS members having been identified in *Arabidopsis thaliana* and rice (*Oryza sativa L.*), respectively (Tian et al., 2004, Lee et al., 2008). Studies on SCL expression have shown that these genes confer abiotic stress to a plant. A gene OsGRAS23 (homologs to *Arabidopsis* AtSCL9 and AtSCL14) was found to be involved in a response to drought stress in rice (Xu et al., 2015). The over-expression of OsGRAS23 is responsible for an enhanced resistance to drought and oxidative stresses through the up-regulation of stress-responsive genes. In *Arabidopsis thaliana*, SCL7 expression increased more than 8-fold in the leaf tissue of plants undergoing salt stress (350 mM NaC, 6 h) (Ma et al., 2010). A novel wheat GRAS gene (TaSCL14) has been shown to regulate plant growth, photosynthesis and photooxidative tolerance (Chen et al., 2015) while SCL3 expression in *Arabidopsis* plants positively regulates and maintains functional GA pathways, which play a key role in the control of processes occurring during root development (Heo et al., 2011, Zhang et al., 2011).

In barley, SCL expression was up-regulated in the leaf tissue of almost all barley cultivars, except for Steptoe barley, which was seen to significantly decrease (FC, -28.57 ± 1.27) and in Arivat barley, where no change was observed (FC, -1.18 ± 0.32). The highest expression of SCL in barley leaf tissue was Gairdner barley (FC, 5.09 ± 0.73), followed by Hindmarsh (FC, 3.15 ± 0.57) and CM72 (FC, 2.51 ± 1.03) barleys. SCL showed a difference in its root tissue expression pattern compared to that of the leaf tissue in all barley cultivars. The Calmariout barley displayed the lowest SCL expression, with a greater than 25-folddecrease, followed by Steptoe (FC, -3.21 ± 0.39) and Hindmarsh (FC, -1.17 ± 1.02) barleys. The remaining cultivars showed an up-regulation of the SCL transcript, with the highest gene expression being recorded for Stirling (FC, 4.82 ± 1.51) barley, followed by Gairdner (FC, 3.98 ± 1.34), Buloke (FC, 2.76 ± 0.71), Arivat (FC, 1.87 ± 0.35) barleys, with the CM72 barley showing almost no change at all (Table 8.4.1; Figure 8.7.1).

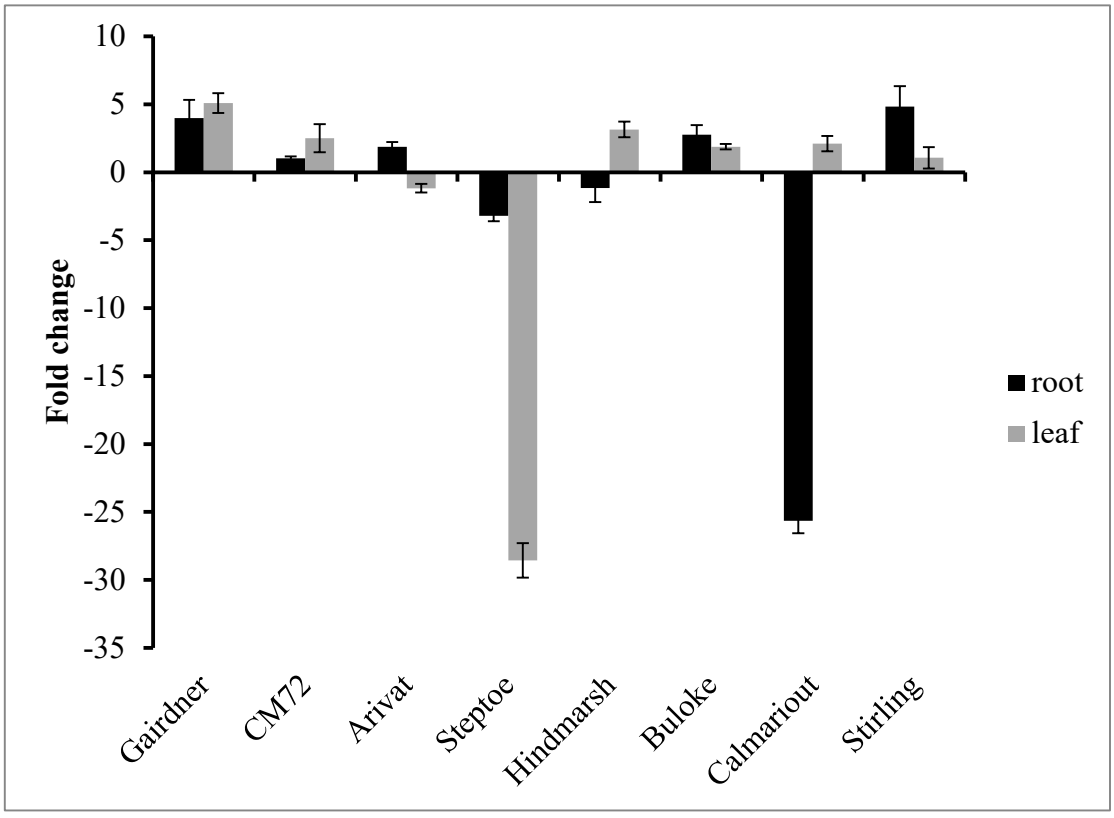

Figure 8.7.1 Differential expression of SCL in the leaf and root tissues of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

SCL expression did not show a strong induction after a 24h treament period in both the root and leaf tissue compared to other genes, wih the exception of the significant change in the SCL expression measured in leaf tissue of Steptoe barley and the root tissue of Calmariout baley. This result is consistent with the data reported in the study by Ma et al. (2010), who noted that SCL7 expression reached a maximum in the leaf tissue of *Arabidopsis* after 3 h of salt treatment, with the expression then decreasing gradually after this time. These results are also consistent with those reported by Shi et al. (2015), who found that the GRAS genes expressed were greatest in the root tissue of upland cotton (*Gossypium hirsutum* L.) after 3 h of salt treatment (150 mM NaCl). This expression then decreased after 12 and 48 h of salt treatment. The difference in the expression pattern could be explained by the presence of a genome-specific gene among the barley varieties.

8.8 Modulation of SPL expression under salinity conditions

The understanding of SPL expression is in its early stages, but it is known that SPL has a number of diverse funtional roles in the growth and development of plants and their response to abiotic stress. A number of SPL expressions have been identified in *Arabidopsis thaliana* (16), *Oryza sativa* (18) and *Zea mays* (31) (Cardon et al., 1999, Hultquist and Dorweiler, 2008, Miura et al., 2010).

As seen from the data presented in Figure 8.8.1, the differential expression of SPL was found to be higher in leaf tissue than in the root tissue of barley after salt treatment. The change in expression ranged from 1.2- to 14.4-fold changes in the leaf tissue, whereas in the root tissue, the expression was much lower, ranging from 1.04 to 2.59-fold change, with the exception of the Calmariout barley root tissue showing a significant change in expression (FC, -19.23 ± 0.30). It can be concluded that the greater SPL expression in the leaf tissue highlighted the sensitivity of this barley variety to response to salt stress, with the leaf tissue showing greater expression than that of the root tissue. These results were consistent with those reported by Wang et al. (2013), who found that a higher differential expression of SPL was observed in the leaves than the roots of cotton (*Gossypium hirsutum* L.) under 0.1 and 0.25% NaCl conditions. These results were also consistent with the work reported by Munns and Termaat (1986), showing that the leaf tissue is more indicative of salt and nutrient

stresses than that of the roots during development. These findings suggest that SPL might be specifically expressed in the leaf tissue of barley.

In leaf tissue, SPL expression showed up-regulation in all of the tested cultivars with the exception of Steptoe barley, where no noticeable change was observed (FC, -1.36 ± 1.27). The highest SPL expression was observed in Gairdner barley (FC, 14.40 ± 0.71), followed by CM72 barley (FC, 8.95 ± 0.88) while a lower expression was observed in the Buloke and Calmariout barleys with FC, 2.52 ± 0.24 and 6.11 ± 0.70, respectively (Table 8.4.1; Figure 8.8.1).

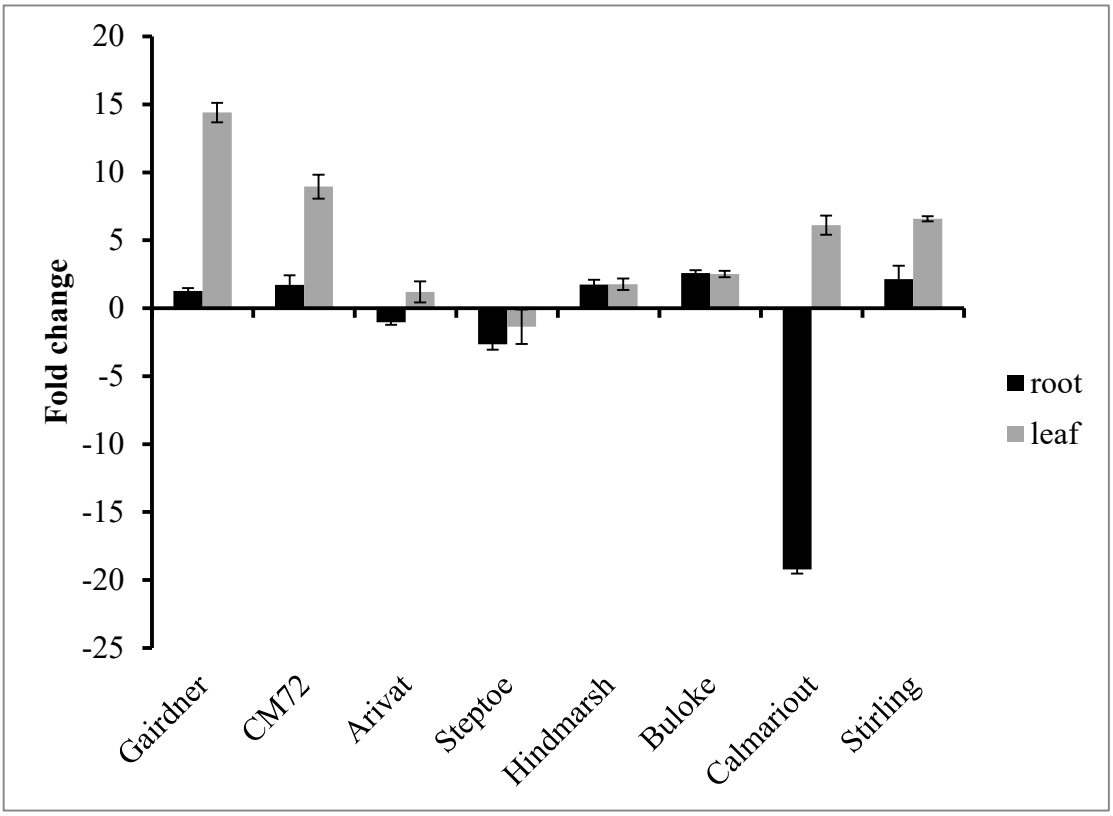

Figure 8.8.1 Differential expression of SPL in the leaf and root tissues of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

In the root tissue, the SPL expression did not show a significant change in all salt resistant groups compared to those of the leaf tissue after salt treatment, with the exception of Calmariout barley, which exhibited a substantial decrease (FC, -19.23 ± 0.30). Expression of SPL in the root tissue was in the same direction as that of the leaf

tissue in almost all samples, however the change was much lower, indicating that SPL may not respond or not have a functional role in the development of roots under acute stress conditions (24 h of 150 mM NaCl). This result is supported by the data provided by Wang et al. (2013), who found that the expression of SPL significantly increased under high salt concentrations (0.5% NaCl for 7 days) and the overexpression of SPL positively regulated the development of leaves, second shoots, and promoted plant flowering (Zhang et al., 2015b).

8.9 Comparative analysis of gene expression

Gene expression has been studied in a variety of plants in order to understand the regulatory mechanism used by plants to respond to salt stress. Numerous genes have been investigated for their differential expression under various stress conditions using real time PCR. In the present study, the expression of six genes (MYB6, BADH1, CMO1, ABI5, SCL and SPL) that have been reported to play an important role in various plant growth and development processes, were investigated. In general, these genes exhibit a range of diverse expression behaviours in both the leaf and root tissue of the barley cultivars being tested. The expression of six genes in the root tissue of barley plants, ranging from salt sensitive to salt tolerant varieties are presented in Figure 8.9.1. Notably SCL expression was significantly down-regulated in the salt-tolerant Calmariout barley variety. SCL expression has been reported to control the extent of root development via the regulation of GA (Heo et al., 2011), indicating that the SCL inhibits root growth in the Calmariout barley, whereas SPL expression is known to act in the process of leaf development (Zhang et al., 2014). This finding supports the role of SCL in the root tissue response to salt stress. In leaf tissue, the gene expression increased in almost all of the cultivars, with the exception of the Steptoe barley, where all of the selected genes were down-regulated under salinity stress condions, suggesting that these genes play a role in responding to exposure to salt stress (Figure 8.9.1).

Based on the FC of $\geq +1.5$ or ≤ -1.5 being indicative of noticeable change, some of genes listed above were not significantly differentially expressed in response to the salt stress conditions, whereas others exhibited a significant degree of differential expression (Table 8.4.1). The expression of genes was also found to vary according to whether the samples were obtained from the leaf or root tissue, and varied

among the various cultivars. The expression of these tested genes in the leaf and root tissue were almost inconsistent with the classification method previously discussed, which was based on a physiological approach. The discrepancy could be due to factors such as tissue specific expression, cultivar specific expresion, expression taking place at specific developmental stages or under special conditions, or co-regulation taking place between the genes being studied. In addition, four out of the six genes tested remained almost unchanged in both the leaf and root tissue of the Arivat barley, whereas the remaining genes exhibited an approximately 2-fold change, suggesting that the Arivat barley may recover to a reduced steady rate of growth after 24 h of salt treatment. This finding support the previous studies that have shown an almost no changed in the RWCI and Na^+/K^+ index in Arivat barley after 24 h of salinity exposure. Among the selected genes, MYB expression was significantly up-regulated in response to salt stress. This gene could be used as an excellent candidate for salt tolerance testing.

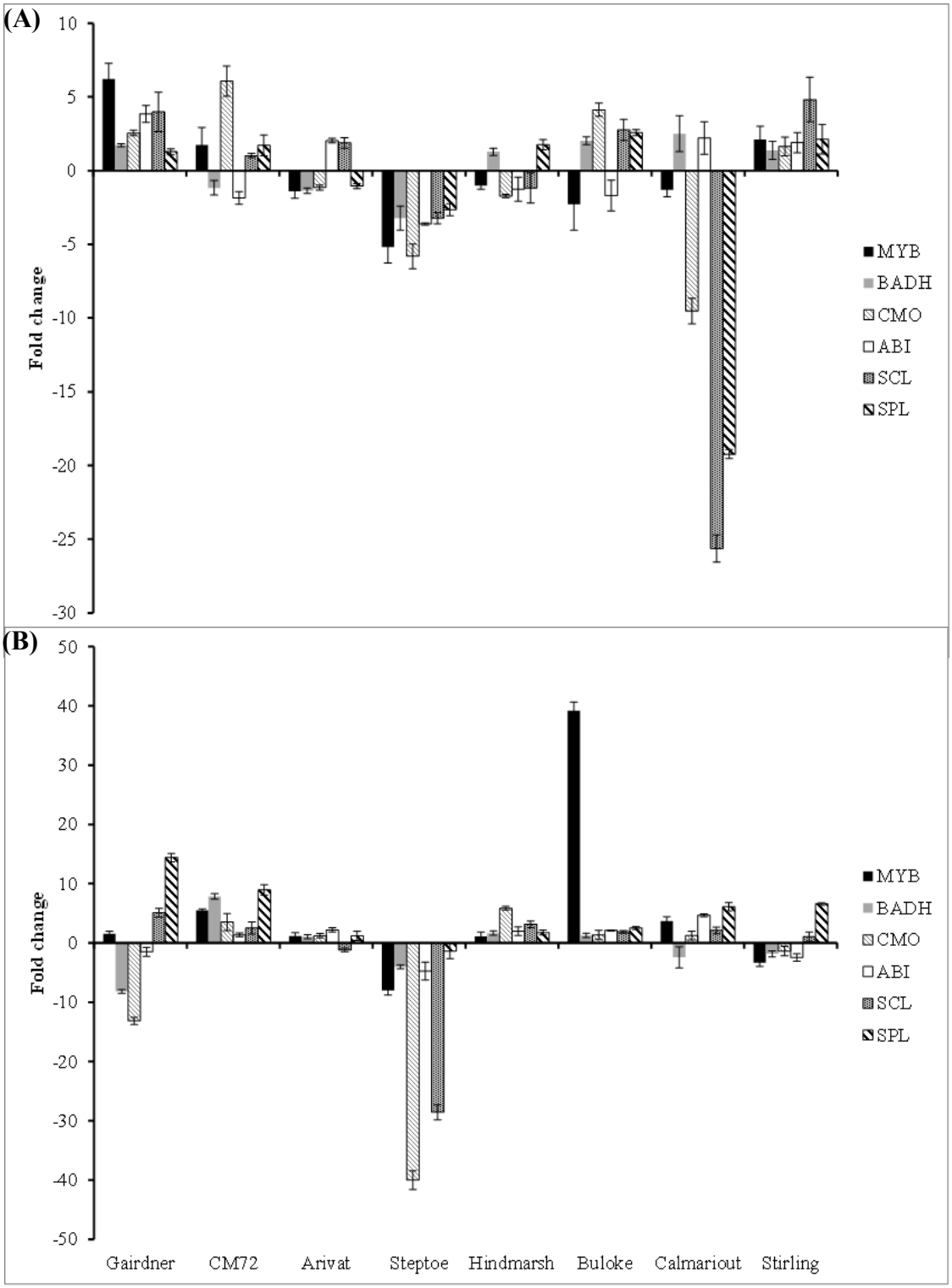

Figure 8.9.1 Differential expression of all six selected genes in the **(A)** root tissue and **(B)** leaf tissue of eight barley cultivars after 24 hours of exposure to 150 mM NaCl. Mean ± SE (n=3).

8.10 Summary

This chapter reported the expression pattern of some functional genes that have been reported to play a role in response to exposing barley plants to abiotic stress. Among the complicated genetic network, six genes were chosen for study in order to examine their expression under salt stress conditions. These genes included MYB6, CMO, BADH, ABI5, SCL and SPL, selected because of their important role in plant developmental processes. Investigation of their expression pattern was carried out under acute stress conditions (150 mM NaCl for 24 hours) in the leaf and root tissue of eight barley varieties (Buloke, Calmariout, Stirling, Arivat, CM72, Steptoe, Hindmarsh and Gairdner barleys), which have been classified as being salt tolerant, salt moderated and salt sensitive according to a physiological classification approach, as discussed in Chapter 7.

Six genes showed notable degrees of modulation of gene expression under salt stress conditions (FC $\geq +1.5$ or ≤ -1.5) in almost all of the tested genotypes. In leaf tissue, the most significant increase was found in the MYB6 gene (FC, 39.2 ± 1.42) while the greatest decrease in expression was found for the CMO gene (FC, -40.00 ± 1.57). In the root tissue, MYB6 expression showed the greatest up-regulation in the Gairdner barley, with an FC of 6.23. SCL expression exhibited the greatest down-regulation in the Steptoe genotype, having an over -28.57-fold change. In general, the gene expression taking place in leaf tissue is greater than that taking place in the root tissue, indicating that the leaf tissue may exhibit greater sensitivity to salinity exposure. Although the genes displayed variations in expression, they showed significant changes in the leaf or root tissue among the tested varieties. This suggests that the MYB6, CMO, BADH, ABI5, SCL and SPL genes might play a role in allowing both leaf and root tissues of barley to confer salt tolerance to the plant. The significant up-regulation of the MYB6 gene in the leaf and root tissue suggests that this gene could be chosen as a suitable candidate for study with a view to improve the degree of salt tolerance.

Chapter 9.

Comparative analysis of miRNAs across bacteria of the genus *Thalassospira* and the barley *Hordeum vulgare* L.

9.1 Overview

As noted in Chapter 2, , miRNAs have been shown to play a critical role in the growth and developmental processes of diverse organisms (Ambros, 2004, Reinhart et al., 2002). It was also demonstrated that plant miRNAs are responsive to environmental stresses, such as salinity, drought and heat stress. The overexpression of miRNAs is a regulatory mechanism, which leads to improved ability to adapt and survive in changing environmental conditions. The occurrence of miRNAs has been investigated in a number of different organisms, of which many have been found to be conserved across evolutionary different species (Campo-Paysaa et al., 2011, Cuperus et al., 2011, Rathore et al., 2016). In plants, many miRNA families were found to be conserved in *Arabidopsis thaliana*, rice, barley, wheat, *Brachypodium*, *Populus*, maize and *Sorghum* (Jones-Rhoades et al., 2006, Yao et al., 2007, Schreiber et al., 2011). Conserved miRNAs have also been identified in different animal lineages (Campo-Paysaa et al., 2011). In this project miRNAs were identified in bacteria of the genus *Thalassospira* and in the barley *Hordeum vulgare* L., with the intent to comparatively evaluate whether or not phylogenetically related stress responsive genes, which are present in halophilic bacteria, could be retrieved in plant species. Several genes, including those exhibiting salt tolerance, such as trehalose, glycine betaine, heat shock like proteins or Na^+ and H^+ antiporters (Das et al., 2015, Rubiano-Labrador et al., 2015, Thombre et al., 2016) were selected for comparative analysis. These studies, therefore, suggests the possibility of miRNA conservation between halophilic bacteria and plants. Given this background, this chapter presents a comparative analysis of the miRNAs identified in bacteria of the genus *Thalassospira* and the barley *Hordeum vulgare* L. in order to assess the degree of phylogenetic relatedness between the miRNA populations of prokaryotic (bacteria) and eukaryotic (plant) organisms.

9.2 Identification of conserved miRNAs in bacteria of the genus *Thalassospira* and barley *Hordeum vulgare* L.

In order to analyse miRNAs sequence relatedness between bacteria of the genus *Thalassospira* and the barley *Hordeum vulgare* L., salt responsive barley miRNAs were aligned against putative bacterial miRNAs using MAFFT (Multiple Alignment using Fast Fourier Transform) program. Sequences having ≥ 50% identity were then analysed again using EMBOSS Stretcher and global alignment function

available in the BLASTN program (Altschul et al., 1997) with default setting. Based on current understanding of miRNA – target mRNA interactions, the miRNAs were considered to be conserved by either having a perfect match of 'seed sequence' at 5' end (6 nucleotides at positions 2-7) (detailed in Chapter 2) or highly similar sequence identity across full-length of miRNAs (> 70% identity) (Lenz et al., 2011, Ibáñez-Ventoso et al., 2008).

9.2.1 5' end seed region criterion

5' end seed sequences play a critical role in the function of miRNA, as the recognition site binds either perfectly, or near perfectly, to target mRNAs (Lewis et al., 2005). This region has also been reported to be highly conserved across animal species (Lewis et al., 2005). Therefore, 5' seed regions were used to search for matches between the salt responsive bacterial and the barley miRNAs identified in this study (as described in Chapters 5 and 6) to satisfy the following conditions: i) at least 7 continuous nucleotides are identified that are completely identical within the first 10 nucleotides of the miRNAs and ii) no mismatches or gaps existing within the first 10 nucleotides, except for changes in base. Twenty-five out of 41 salt responsive barley miRNAs were found to be homologous in the seed sequences of putative bacterial miRNAs, while no conservation was identified in the remaining 14 barley miRNAs (Table 9.2.1). Most of the barley miRNAs possessed conserved 5' end seed sequences to one of the studied bacterial miRNAs species; some of them shared seed regions that were conserved to miRNAs identified in different species. The presence of the conserved 5' end seed regions suggested that these conserved miRNAs have been involved in similar processes.

Table 9.2.1 5’ end seed sequence conservation between salt responsive bacterial and barley miRNAs

5’ sequence-related miRNAs		
Bacteria	**Barley**	**Sequence alignment**
T.luce_5p_132929	hvu-MIR171	hvu-MIR171 -TGTTGGCTCGACTCACTCAGA T.luce_5p_132929 ATGTTGGCTTTGCCGTTGCTGG
T.aust_3p_9648 T.xian_5p_16719	hvu-MIR159a/b	T.xian_5p_16719 ATTTTGGATGCCTTGCGTGTTT- hvu-MIR159a/b --TTTGGATTGAAGGGAGCTCTG T.aust_3p_9648 -CTTTGGATTTGTCGGCAAACGC
T.xiam_5p_12105	hvu-MIR5048a/b	hvu-MIR5048a TATTTGCAGGTTTTAGGTCTAA- T.xiam_5p_12105 -ATTTGCATGCCC---GTCTGGC
T. profu_3p_56542	SUT_hvu_mir_000108	SUT_hvu_mir_000108 -CACGAGGGCTCTGCTCGCTGAT T.profu_3p_56542 TGCCGAGGGCCCGCGTGC-GGTC
T. xian_3p_22216	SUT_hvu_mir_000045	SUT_hvu_mir_000045 --GCTTCTTGCTGATGGTGTTATTCC T.xian_3p_22216 GGGCTTCTTTTTTGCCGGTCG----
T.luce_5p_37686	SUT_hvu_mir_000163	SUT_hvu_mir_000163 CATATATGTAGTGCTGTAAGAAGA T.luce_5p_37686 CGTATATGTCACACGGCATGTG—
T.xian_5p_16719	SUT_hvu_mir_000150	SUT_hvu_mir_000150 --TTTGGATCGAAGGGAGTTTTTT T.xian_5p_16719 ATTTTGGATGCCTTGC-GTGTTT-
T.luce_5p_248131	SUT_hvu_mir_000133	SUT_hvu_mir_000133 GAACGATTTGAGGCGATTTGAAC- T.luce_5p_248131 --ACGATTTTGTCGGCCATGGTCA
T.profu_5p_15457	SUT_hvu_mir_000075	SUT_hvu_mir_000075 ---AAGGAAACTGGGGCAGTGGCATAT T.profu_5p_15457 CGCAAGGAAAAGCCCCGCAGAC------

5’ sequence-related miRNAs		
Bacteria	**Barley**	**Sequence alignment**
T.luce_3p_171055	SUT_hvu_mir_000050	SUT_hvu_mir_000050 AAACAGATCTCAAGGATCTATT- T.luce_3p_171055 -AACAGATCAAGTGACGGTGCTT
T.luce_3p_522493	SUT_hvu_mir_000127	SUT_hvu_mir_000127 CTTGCTCCCTTTCATTTTTTGT- T.luce_3p_522493 -ACGCTCCCTGCTTCGACACTTA
T.luce_3p_252720	SUT_hvu_mir_000029	SUT_hvu_mir_000029 CGCCGTCGCTTCGTCGTACATC T.luce_3p_252720 CGCCGTCAGGATGCCGCGGGCA
T.luce_5p_346590	SUT_hvu_mir_000025	SUT_hvu_mir_000025 -GTGCTTATTGACGGTCCAGTGCT T.luce_5p_346590 CGGGCTTATTTCATGAGTCCGG--
T.luce_5p_346590	SUT_hvu_mir_000026	SUT_hvu_mir_000026 -GAGCTTATTGACGGTCCAGTGCT T.luce_5p_346590 CGGGCTTATTTCATGAGTCCGG--
T.tepi_3p_11264	SUT_hvu_mir_000027	SUT_hvu_mir_000027 ATGGGATTGCTCGTATTATAGGTC T.tepi_3p_11264 -AAGGATTGCGGTCGGCCTTACT-
T.profu_3p_28449	SUT_hvu_mir_000039	SUT_hvu_mir_000039 TGAATTTGTTTAACTAGAAT-TTAT T.profu_3p_28449 ---ATTTGTTTCAGGCATAGGACAT
T.aust_3p_4053 T.luce_3p_31255 T.luce_3p_82704	SUT_hvu_mir_000228	SUT_hvu_mir_000228 TTTGCCATCAGCCTTGGGGCT-- T.aust_3p_4053 -TTGCCATCCCGATTGCAATTGA SUT_hvu_mir_000228 --TTTGCCATCAGCCTTGGGGCT T.luce_3p_31255 GGTTTGCCAATGGGCAGAAACC- SUT_hvu_mir_000228 TTTGCCATCAGC--CTTGGGGCT- T.luce_3p_82704 --TGCCATCGAAGCCCTTTCGGTG

5’ sequence-related miRNAs		
Bacteria	**Barley**	**Sequence alignment**
T.aust_3p_33837	SUT_hvu_mir_000079	SUT_hvu_mir_000079 AAGTTGGGCAATAATGTTGTA-- T.aust_3p_33837 -TTTTGGGCAAGGATGCGGTTGA
T.tepi_3p_17794 T.profu_5p_11482	SUT_hvu_mir_000041	SUT_hvu_mir_000041 TTGGCGGAGCTCCTGCCCTATTT T.tepi_3p_17794 TTGGCGGTGATCTTCATGACTT- SUT_hvu_mir_000041 -TTGGCGGAGCTCCTGCCCTATTT T.profu_5p_11482 TTTGGCGGTTTCT-CGCCCGAGG
T.profu_3p_5000	SUT_hvu_mir_000062	SUT_hvu_mir_000062 GCGAACGAACGATCTAAACT-- T.profu_3p_5000 GGTAACGAACTGTAATTACACT
T.luce_3p_528636	SUT_hvu_mir_000186	SUT_hvu_mir_000186 TGGCGCTCCTGCTGCGCTCTCC- T.luce_3p_528636 -TGCGCTCCGATGCGCGTGAACC
T.aust_5p_23871 T.luce_5p_585272	SUT_hvu_mir_000061	SUT_hvu_mir_000061 TAGGTTCATCCGTTGTCGCT-- T.aust_5p_23871 AACGTTCATCGTTTGGAACGTT T.luce_5p_585272 TGAGTTCATCAAGTGCGCGGCG
T.luce_5p_210055	SUT_hvu_mir_000043	SUT_hvu_mir_000043 --GGCGGATG-TAGCCAAGTTGAG T.luce_5p_210055 TCGGCGGATTTTTGACCCGATT--

The matching seed sequences were highlighted in grey.

9.2.2 Full-span homology over miRNA criterion

Due to the preference of binding to target transcripts over the length of some miRNAs instead of the less stringent 5' seed pairing technique (Brennecke et al., 2005, Doench and Sharp, 2004), the criterion of measuring the homology over the entire miRNA length was applied to determine the degree of miRNA relatedness (Ibáñez-Ventoso et al., 2008). Analysis of the salt responsive barley miRNAs that had been aligned against the full length of putative bacterial miRNAs revealed that all 41 barley miRNAs that were detected shared sequence similarity to that of the bacterial miRNAs, with the highest similarity being found to the SUT_hvu_mir_000075 and T.luce_5p_525349 (68% identity) (Table 9.2.2; Appendix 2). As seen in the Table 9.2.2, putative bacterial miRNAs had the most number of sequence similarity to SUT_hvu_mir_000186 with 21 sequences having ≥ 50% identity, following by SUT_hvu_mir_000049 with 18 sequences and SUT_hvu_mir_000084 with 17 sequences while the least number of sequence similarity belonged to SUT_hvu_mir_000142 with ≥ 50% identity to only 3 putative bacterial miRNAs. Sequence alignment was carried out by EMBOSS Stretcher and BLASTN global alignment programs which gave the same percent identity, confirmed the significant reliability of the analysis. Based on the cut-off value of >70% identity over miRNA sequence length that had been established by Ibáñez-Ventoso et al. (2008) for the identification of miRNA homologs known from published sites or miRNA groups, the phylogenetic relatedness between the barley and bacterial miRNAs was found to be much lower. While the 70% cut-off value had been previously applied in analysis of miRNAs that were conserved across the phylogenetically more closely related species of eukaryotes, *e.g*., plant species (Lenz et al., 2011) or animal species (Ibáñez-Ventoso et al., 2008), it appears that no comparative phylogenetic analysis of conserved miRNAs between prokaryotes and eukaryotes has been conducted until the current study. It is therefore proposed that ≥50% similarity of conserved miRNAs could be applied as a criterion for classification of phylogenetically distant organisms, such as bacteria and plants. Furthermore, the conserved miRNA sequences that were found to be identical in bacteria and plants suggests their function similarities may have arisen from their common evolutionary development (Kovalchuk and Kovalchuk, 2012).

Table 9.2.2 Putative miRNAs of bacteria of the genus *Thalassospira* with ≥ 50% similarity to those of salt responsive barley miRNAs.

Full sequence-related miRNAs		% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**	
T. luce_5p_21030	hvu-MIR171	50.0
T.luce_3p_252989		59.0
T. aust_3p_39387		50.0
T. luce_5p_181596		54.5
T.tepi_5p_9402		50.0
T. profu_3p_56451	hvu-MIR159a/b	50.0
T. profu_3p_38494		50.0
T. luce_3p_432525		54.5
T.xian_3p_3489		54.5
T.luce_3p_602433		54.5
T.aust_3p_9648		50.0
T.aust_5p_57006		54.5
T.tepi_3p_19433/ T.profu_3p_29989		50.0
T.profu_3p_33610	hvu-MIR5048a/b	54.5
T.profu_5p_56952		54.5
T.xian_3p_22881		50.0
T.xian_5p_16719		50.0
T.aust_3p_3291		54.5
T.luce_3p_249012		50.0
T.xiam_3p_34788		54.5
T.xiam_5p_17371		50.0
T.tepi_3p_34995		54.5
T.xiam_3p_38869		50.0
T.aust_3p_33837		56.5
T.alka_5p_329	SUT_hvu_mir_000173	54.5
T.profu_5p_7879		50.0
T.profu_5p_3800		50.0
T.xian_5p_25207		52.2
T.luce_3p_523350		50.0
T.luce_5p_544598		50.0

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T.luce_5p_589692		50.0
T. luce_5p_617474		50.0
T.tepi_5p_5471		50.0
T.profu_5p_15457	SUT_hvu_mir_000108	50.0
T.xian_3p_19488		50.0
T.luce_3p_198770		54.5
T.luce_5p_22792		50.0
T.luce_3p_235876		50.0
T.aust_3p_16396		50.0
T.xiam_3p_12450		50.0
T.aust_5p_7801		52.2
T.profu_3p_44551	SUT_hvu_mir_000174	58.3
T.luce_5p_528636		52.2
T.luce_5p_38961		52.2
T.luce_5p_531055		60.9
T.luce_3p_544598		52.2
T. luce_3p_306227		52.2
T.luce_3p_132929		52.2
T.tepi_5p_17561		52.2
T.luce_5p_528656/ T.luce_5p_528636		52.2
T.profu_3p_778/ T.tepi_3p_572	SUT_hvu_mir_000045	54.2
T.profu_3p_24235/ T.tepi_3p_15757		54.2
T.xian_3p_19488		54.2
T.xian_3p_3333		54.2
T.luce_3p_394944		50.0
T.aust_5p_54156		60.0
T.aust_3p_53512		54.2
T.tepi_3p_8120		50.0
T.xiam_3p_34788		50.0
T.tepi_3p_21300		50.0
T.xian_3p_16668/ T.xiam_3p_26826		50.0
T. tepi_3p_7722		54.2

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T.alka_3p_4942/T.luce_3p_520914	SUT_hvu_mir_000163	58.3
T.profu_3p_30109/T.tepi_3p_19555		50.0
T.profu_5p_32260/T.tepi_5p_20938		60.0
T.luce_3p_520914		58.3
T.xian_3p_22881		50.0
T.luce_3p_314181		54.2
T.luce_3p_546117		50.0
T.aust_5p_40281		50.0
T.tepi_5p_29436		54.2
T.tepi_5p_34995		50.0
T. profu_5p_44441	SUT_hvu_mir_000150	52.2
T.luce_3p_14713		54.5
T. xian_5p_23642		50.0
T. luce_3p_350700		54.5
T.profu_5p_50983		50.0
T.luce_5p_528656/ T.luce_5p_528636		54.5
T.luce_3p_312757		50.0
T.luce_5p_186751		54.5
T.luce_3p_549646		50.0
T.tepi_5p_8741		50.0
T.tepi_3p_19094		54.5
T.aust_5p_30915		50.0
T.profu_5p_43344	SUT_hvu_mir_000133	60.9
T.profu_5p_44486		60.9
T.luce_3p_384973		52.2
T.xian_3p_16719		56.5
T.luce_5p_167793		52.2
T.luce_3p_314181		52.2
T.luce_3p_498538		52.2
T.luce_3p_354019		52.2
T.luce_5p_602433		58.3
T. profu_5p_9602	SUT_hvu_mir_000075	50.0

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T.alka_5p_4279		50.0
T.luce_5p_525349		68.0
T.luce_3p_58702		50.0
T.luce_3p_559495		50.0
T.luce_5p_399890		54.2
T.tepi_5p_24769		56.0
T.alka_3p_4279	SUT_hvu_mir_000049	54.5
T.alka_3p_1819		50.0
T. luce_3p_399471		50.0
T.profu_5p_12216		50.0
T.profu_5p_50983		54.5
T.luce_5p_528656/ T. luce_5p_528636		54.5
T.luce_3p_559608		50.0
T. xian_5p_22099		50.0
T. luce_3p_218949		50.0
T.luce_5p_167793		50.0
T.aust_5p_42495		50.0
T.luce_3p_422620		62.5
T.aust_5p_3291		54.5
T.aust_3p_52437		50.0
T.tepi_5p_8405		50.0
T.profu_3p_37885/ T.tepi_3p_24769		56.6
T.tepi_3p_33254		50.0
T.aust_5p_9684		50.0
T. profu_5p_33977	SUT_hvu_mir_000050	63.6
T. profu_3p_54600		50.0
T. xian_3p_3489		50.0
T. luce_5p_399471		50.0
T.luce_5p_252989		63.6
T.luce_3p_617474		50.0
T.luce_5p_481971		50.0
T.luce_3p_609984		50.0

Full sequence-related miRNAs		% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**	
T.aust_5p_58885		54.5
T.aust_5p_16396		50.0
T. luce_5p_678		50.0
T. luce_3p_678		50.0
T. aust_5p_3299		50.0
T. xiam_5p_8112		50.0
T.profu_5p_28449	SUT_hvu_mir_000081	50.0
T.profu_5p_17011		50.0
T.profu_5p_30109		50.0
T.xian_3p_19710		54.5
T.profu_5p_50983		50.0
T.aust_5p_968		52.2
T. tepi_5p_19555		50.0
T.profu_3p_24901	SUT_hvu_mir_000127	59.1
T.profu_3p_33610		65.2
T.profu_3p_33977		50.0
T.luce_3p_338996		50.0
T.luce_3p_273440		50.0
T.xian_3p_16407		50.0
T.luce_3p_198770		50.0
T.aust_3p_56497		50.0
T.tepi_3p_6580		59.1
T.xiam_3p_34788		54.5
T.tepi_3p_23682		52.2
T.tepi_3p_11218		50.0
T.profu_5p_49438/ T.tepi_5p_33046	SUT_hvu_mir_000029	54.5
T.profu_3p_9602/ T.tepi_3p_6592		50.0
T.profu_5p_45214		50.0
T.profu_3p_18810		50.0
T.aust_3p_10018		50.0
T.luce_3p_471094		59.1
T.luce_3p_48943		65.2

Full sequence-related miRNAs		% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**	
T.luce_3p_529459		50.0
T.xian_3p_3333		50.0
T.luce_5p_282136		50.0
T.aust_3p_15365		50.0
T.luce_3p_43186		50.0
T.luce_3p_585272		50.0
T.aust_3p_47340		50.0
T.tepi_3p_8120		50.0
T.aust_5p_22981		54.5
T.prof_5p_19418	SUT_hvu_mir_000025	50.0
T.profu_3p_21694		52.2
T.xian_3p_20844		56.5
T.luce_3p_556182		52.2
T.xian_5p_3333		56.5
T.luce_3p_612357		52.2
T.luce_3p_608572		52.2
T.aust_5p_39387		56.5
T.aust_3p_37611		54.2
T.pova_5p_489	SUT_hvu_mir_000095	54.5
T.profu_3p_32260		50.0
T.alka_3p_1023		50.0
T.luce_5p_31831		50.0
T.prof_3p_49		54.5
T.luce_5p_156594		54.5
T. luce_3p_302533		50.0
T.luce_5p_65195		50.0
T.luce_5p_354019		50.0
T.aust_3p_7801		50.0
T.tepi_3p_20938		50.0
T.pova_3p_2290	SUT_hvu_mir_000026	56.5
T.luce_3p_464921		52.2
T.xian_3p_20844		56.5

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T.xian_5p_22881		52.2
T.luce_3p_556182		52.2
T.aust_5p_57006		54.2
T.xian_3p_32022	SUT_hvu_mir_000158	54.2
T.aust_3p_56957		50.0
T.luce_5p_23450		50.0
T.luce_3p_531055		54.2
T.tepi_3p_28590		50.0
T.profu_5p_43344	SUT_hvu_mir_000084	50.0
T.meso_3p_3026		54.5
T.profu_5p_44486		50.0
T.profu_3p_24324		50.0
T.profu_5p_18148		50.0
T.xian_5p_17518		65.2
T.tepi_5p_8405		50.0
T.profu_5p_12074		50.0
T.profu_3p_56542		50.0
T.aust_3p_11614		50.0
T.aust_5p_40281		58.3
T.tepi_3p_30879		58.3
T.aust_5p_53204		58.3
T.aust_5p_28718		54.2
T.tepi_3p_3446		62.5
T.tepi_3p_33254		50.0
T.tepi_5p_7722		50.0
T.alka_5p_4684	SUT_hvu_mir_000112	54.2
T.xiam_3p_34788		56.5
T.tepi_3p_18168		52.2
T.profu_3p_32508	SUT_hvu_mir_000027	54.2
T.pova_5p_390		50.0
T. xian_3p_6822		50.0
T.xian_3p_18366		50.0

Full sequence-related miRNAs		% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**	
T.aust_3p_54777		50.0
T.aust_3p_58885		54.2
T. luce_3p_474240		50.0
T.aust_5p_39756		54.2
T.luce_3p_559495		52.0
T.luce_3p_421879		60.0
T.tepi_5p_11782		50.0
T.tepi_3p_22942		54.2
T.xiam_3p_6543		54.2
T.profu_3p_38494	SUT_hvu_mir_000142	52.2
T.profu_5p_51572		50.0
T.luce_3p_599500		54.5
T.alka_3p_3811	SUT_hvu_mir_000046	50.0
T.profu_3p_20856		54.5
T.xian_3p_19710		50.0
T.aust_5p_13346		54.5
T.luce_3p_601689		50.0
T.luce_3p_556182		50.0
T.luce_5p_385818		50.0
T.xian_5p_20844		50.0
T.luce_5p_593263		54.5
T.luce_3p_354019		54.5
T.aust_5p_5027		50.0
T.aust_5p_32539		50.0
T.xiam_5p_35854		50.0
T.alka_5p_1519	SUT_hvu_mir_000023	50.0
T.alka_5p_5116		50.0
T.luce_5p_210055		50.0
T. aust_3p_20162		50.0
T.aust_3p_56497		54.5
T.luce_3p_44717		50.0
T.tepi_3p_9218		50.0

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T.tepi_3p_33254		50.0
T.aust_3p_968		52.2
T.pova_5p_489	SUT_hvu_mir_000094	63.5
T.profu_5p_57479		59.1
T.profu_3p_12216		50.0
T.xian_5p_17681		50.0
T.aust_5p_39387		50.0
T.luce_5p_585272		50.0
T.alka_5p_4942/ T.luce_5p_520914	SUT_hvu_mir_000039	54.2
T.profu_3p_56451		58.3
T.profu_3p_50983		54.2
T.xian_5p_9958		50.0
T.aust_3p_3291		50.0
T.aust_5p_47340		54.2
T.tepi_5p_33271		54.2
T.profu_3p_14407	SUT_hvu_mir_000034	50.0
T.alka_5p_3838		50.0
T.profu_5p_24324		50.0
T.luce_5p_82704		50.0
T.luce_3p_82704		50.0
T.profu_5p_21694		65.2
T. aust_3p_23133		50.0
T.luce_5p_246051		50.0
T.aust_3p_36180		50.0
T.alka_5p_3811	SUT_hvu_mir_000228	54.5
T.alka_5p_4684		54.5
T.profu_3p_51013		56.5
T.profu_3p_28449		54.5
T.profu_3p_54785		50.0
T.profu_5p_24235		59.1
T.xian_3p_3512		54.5
T. aust_5p_2750		54.5

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T.aust_5p_17456		54.5
T.aust_5p_7549		54.5
T.profu_3p_49438/T.tepi_3p_33046	SUT_hvu_mir_000079	54.5
T.alka_5p_333		50.0
T.alka_3p_4120		54.5
T.profu_3p_38494		59.1
T.luce_5p_543369		56.5
T.luce_3p_285535		60.9
T.luce_3p_406605		50.0
T.aust_3p_63319		54.5
T.aust_3p_53294		52.4
T.xian_5p_9480	SUT_hvu_mir_000180	56.0
T.profu_3p_5000		50.0
T.luce_5p_285535		58.3
T.aust_3p_49513		50.0
T.tepi_3p_22942		54.2
T.luce_3p_14713	SUT_hvu_mir_000041	52.2
T.profu_3p_41148		52.2
T. luce_5p_48943		56.5
T. luce_5p_210055		60.9
T. luce_3p_559608		58.3
T. xian_5p_16719		52.2
T. luce_5p_637434		56.5
T. luce_3p_385818		52.2
T.luce_3p_8020		56.5
T. luce_3p_132929		56.5
T. aust_3p_57940		60.9
T.xiam_5p_37696		52.2
T. tepi_3p_8405		60.9
T.profu_5p_24901	SUT_hvu_mir_000135	50.0
T. profu_5p_15172		50.0
T.xian_3p_20546		50.0

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T.xian_3p_32022		50.0
T.profu_3p_21694/T.tepi_3p_13698		50.0
T.xian_5p_16719		50.0
T.aust_5p_36180		54.5
T. luce_5p_316125		50.0
T. luce_5p_394944		50.0
T.luce_5p_465632		50.0
T.luce_5p_235876		50.0
T. luce_3p_520914	SUT_hvu_mir_000062	50.0
T. profu_3p_5000		63.6
T. luce_3p_282136		50.0
T. tepi_3p_20938		50.0
T.profu_3p_33954	SUT_hvu_mir_000186	54.5
T. alka_3p _2265		59.1
T.profu_3p_37885/T.tepi_3p_24769		50.0
T.profu_3p_7879/T.tepi_3p_5471		50.0
T.profu_5p_49903		50.0
T.profu_5p_18810		54.5
T.tepi_5p_16607		54.5
T.aust_5p_26441		54.5
T.tepi_5p_11638		50.0
T.meso_3p_3086		63.6
T. luce_5p_56287		63.6
T.luce_5p_389406/T.luce_5p_389408		50.0
T. xian_3p_22099		50.0
T. luce_5p_522493		59.1
T. luce_5p_181596		59.1
T. luce_3p_246818		50.0
T. aust_3p_39387		54.5
T.luce_5p_531055		50.0
T.tepi_3p_30879		50.0
T.aust_3p_7549		54.5

Full sequence-related miRNAs		% identity
Bacterial miRNAs	**Barley miRNAs**	**(Stretcher and BLAST)**
T. aust_3p_34002		50.0
T. alka_5p_333	SUT_hvu_mir_000061	50.0
T.pova_5p_489		54.5
T. luce_5p_39407		54.5
T. xian_5p_19488		63.6
T. luce_5p_245911		50.0
T. aust_5p_56957		50.0
T.luce_5p_235876		54.5
T.luce_5p_585272		54.5
T. luce_5p_502941		59.1
T. alka_3p_3149	SUT_hvu_mir_000043	54.5
T.profu_5p_44486		54.5
T.aust_5p_20162		54.5
T. tepi_5p_29936		50.0
T.aust_3p_64344		52.2
T.tepi_5p_2711		56.5
T.tepi_5p_13698		54.5
T.alka_5p_5176	SUT_hvu_mir_000010	54.5
T.tepi_5p_21088		56.5
T. profu_5p_24235		54.5
T.alka_3p_2121		57.1
T.xiam_5p_12633		55.0
T. luce_5p_608218		56.5
T. xian_3p_9958		54.5
T. luce_3p_384973		59.1
T. profu_5p_17170		52.1
T.profu_5p_30378		54.5
T.luce_3p_186751		52.2
T.luce_5p_31255		54.5
T.aust_3p_9648		50.0
T. aust_3p_23871		56.5

9.2.3 Comparative analysis of miRNAs of bacteria of the genus *Thalassospira* and barley *Hordeum vulgare* L.

A comparative analysis of the conserved miRNA of bacteria and plants has not been previously reported. This study provides an overview of the sequence relatedness that exist between prokaryotic and eukaryotic miRNAs, based on their conserved 5' end seed sequences and full-length miRNA sequences. Analysis of the results obtained in this study demonstrates that not all miRNAs shared conserved sequences in both 5' end and full-length similarity criteria. A similar observation was also made in the analysis of conserved miRNAs in *C. elegans, D. melanogaster* and human microRNAs (Ibáñez-Ventoso et al., 2008). It was found that most of the salt responsive barley miRNAs exhibited a high similarity in the seed sequences at the 5' end to bacterial miRNAs, while the homology over the entire miRNA length was found to be lower than that reported for phylogenetically more closely related organisms (> 70% identity) (Ibáñez-Ventoso et al., 2008). Overall, the results obtained in this study indicated the presence of conserved miRNA sequences that were common for plants (barley *Hordeum vulgare* L.) and bacteria (*Thalassospira*). Notably, it was found that two salt responsive miRNAs present in barley possessed shared conserved sequences in both the 5' end seed region and in the full-span homology over the entire miRNA length (Table 9.2.3). Although the average sequence similarity over the entire miRNAs length was not high (50 and 54.5% identity), these similar miRNA sequences, together with the high homology in the seed region, are highly conserved. This is most likely due to these species having similar potential functions, despite them being present in phylogenetically distant organisms (Ibáñez-Ventoso et al., 2008).

Table 9.2.3 miRNAs shared 5' seed sequence* and full-length sequence homology between bacteria of the genus *Thalassospira* and barley.

Sequence-related miRNAs		
Barley miRNAs	**Bacterial miRNAs**	**Full sequence (% identity)**
hvu-MIR159a/b	T.aust_3p_9648	50.0
SUT_hvu_mir_000061	T.luce_5p_585272	54.5

*, indicated 5’ seed sequence homology between miRNAs of bacteria of the genus *Thalassospira* and barley plant.

9.4 Summary

In this chapter, a comparative analysis of miRNAs in the bacteria of the genus *Thalassospira* and barley *Hordeum vulgare* L. was presented for the first time. Alignment of salt responsive barley miRNAs against bacterial miRNAs revealed that most of the barley miRNAs exhibited a high seed sequence identity at the 5’ end, with all of them sharing a sequence similarity over the full-length of the miRNA (≤ 68%). Remarkably, two out of the 41 barley miRNAs were found to share the conserved sequences in both homology criteria, 5’ end seed region and full-span homology over the entire miRNA length. The data reported in this chapter could be used to further facilitate understanding of the evolution of miRNAs and their physiological functions across the organic world kingdoms.

Chapter 10.

Summary and future directions

10.1 Overall summary

The occurrence and analysis of bacterial miRNAs has involved studies that have focused on pathogenic bacteria (Lee and Hong, 2012, Kang et al., 2013). In this thesis, the bacterial miRNAs of environmental bacteria of the genus *Thalassospira* have been studied for the first time. Bacteria of all validly published species belonging to the genus were collected, including the newly descried species *T. australica.* This bacterium, designated NP 3b2^T, was recovered from a sea water sample and stored in the Culture Collection of Marine Microorganisms at Swinburne University of Technology. This bacterium was comprehensively characterised by conducting an analysis of its physiological, biochemical and chemotaxonomic properties, together with a genotypic analysis of the bacterial DNA-DNA hybridisation and through whole genome sequencing.

Next generation, small RNA-sequencing was employed for the identification of miRNAs in nine species, including *Thalassospira australica* NP 3b2^T, *T. alkalitolerans* JCM 18968^T, *T. lucentensis* QMT2^T, *T. mesophila* JCM 18969^T, *T. povalilytica* Zumi 95^T, *T. profundimaris* WP0211^T, *T. tepidiphila* 1-1B^T, *T. xiamenensis* M-5^T and *T. xianhensis* P-4^T. In an attempt to detect miRNAs, several methodological approaches (miRDeep2, CID-miRNA and *MatureBayes*) were employed to predict the potential genes, which resulted in the identification of 984 putative miRNA candidates from nine species (86 from miRDeep2 and 898 from CID-miRNA and *MatureBayse*).

The putative bacterial miRNAs identified for genus *Thalassospira* are presented in this study for the first time. Fifty-seven conserved putative miRNA sequences were found in the species being studied, with six identical sequences being found at different genomic locations in some species, suggesting the presence of a characteristic pattern to the genus miRNA sequences. The high expression level of these putative miRNAs suggested that these potential miRNA species may play a specific physiological role in the growth and development of bacteria of this taxon. Further analysis of these putative miRNAs revealed that they were different to those previously reported for *E. coli* DH10B and *S. mutans* ATCC 25175.

While the miRNAs present in barley *Hordeum vulgare* L. have been investigated under different abiotic stress conditions such as drought, boron or heat stresses (Ozhuner et al., 2013, Kruszka et al., 2014, Kantar et al., 2010), identification of the response of these miRNAs to salinity had not been subsequently reported. The identification of barley miRNAs was carried out using the data obtained from sequencing the RNA from both the control and salt-stressed leaves of two distinct barley genotypes, the Arivat and Calmariout cultivars. Using the Mireap software, 231 miRNAs were identified in both cultivars, of which 41 mature miRNAs were found to be salt responsive with p value < 0.05. Further analysis resulted in the identification of five known, 11 orthologs and 25 novel candidates. Furthermore, 68 target genes were predicted using the psRNA Target Server. Some identified miRNAs and their targets were further validated by cloning/sequencing and qPCR. The difference in the miRNA expression in two barley genotypes may be related to their differing salt resistance abilities.

In order to investigate the potential function of some of the miRNA targets, target genes of conserved miRNAs were selected to examine their expression pattern with previously reported salt responsive genes found in barley cultivars grown under salinity conditions. Therefore, a number of barley varieties that differ in their salt resistance were chosen for a gene expression test. Based on the relative water content and Na^+/K^+ ratio, 15 barley varieties were evaluated for their salinity tolerance at two different conditions (150 mM NaCl for 6 h and 24 h). The results provided a significant variation in their degree of salt resistance through the measurement of their physiological response. The Calmariout and Stirling cultivars were found to be the most salt tolerant, whereas the Gairdner, Morex and Vlamingh cultivars were categorised as being salt sensitive among those tested.

Six genes were then selected for investigation of their expression, in response to salinity in eight barley cultivars of varying salt resistance, using real-time PCR. The results showed i) a significant degree of differential expression of these genes in the genotypes being tested, ii) a high variation existed in the extent of gene expression between cultivars, iii) a greater expression was observed in the leaf tissue compared to those in the root tissue, iv) the MYB6 gene was found to be the highest up-regulator

in the leaf and root tissue, suggesting that this gene could be a suitable candidate for improvement of salt tolerant plants through genetic modification or breeding.

Finally, a comparative phylogenetic analysis of the miRNAs of bacteria of the genus *Thalassospira* and the barley *Hordeum vulgare* L. was performed for the first time. Based on the homology to 5' end seed sequence and full-length mature miRNA sequence criteria, all salt responsive barley miRNAs detected in this study showed a sequence similarity to putative bacterial miRNAs (≥50% identity), with high degrees of conservation in seed region at the 5' end of most tested sequences at 70 % cut-off values. The results provided evidence of homologous miRNAs being present in environmental prokaryotic organisms such as bacteria of the genus *Thalassospira* and plants such as barley *Hordeum vulgare* L.

10.2 Future directions

This project has provided evidence of the presence of putative miRNAs in environmental bacteria, and these putative miRNAs were computationally identified. Additional research needs to be carried out to validate the occurrence of the putative miRNAs detected on bacterial genomes using qRT-PCR and/or the Northern Blot technique. In addition, the high expression of putative miRNAs found in this work suggest that they have a functional role in the life of bacteria. In order to select good miRNA candidates for measuring the salt stress response in halophilic bacteria, future work could be carried out to determine the expression of bacterial miRNAs in response to different salt concentration stresses at different time-points. It would also be beneficial to identify the potential target mRNAs of these putative bacterial miRNAs as this would assist in identifying the function of miRNAs in the life of a bacterium. Bioinformatics tools that could be employed to predict the target mRNAs include TargetRNA, sRNATarget, IntaRNA, RNApredator or psRNA Target Server. This research direction would be greatly facilitated by the availability of the whole genome sequence of bacteria. This could provide a better understanding of the regulatory mechanism of miRNAs, which could assist in the future improvement of the desirable traits.

In term of barley miRNA identification, 41 conserved and novel salt responsive miRNAs, together with their target mRNAs were identified in two distinct barley

genotypes. Future work needs to be carried out to investigate the salt tolerant response arising from highly expressed barley miRNAs, and to determine their targets using transgenic expression studies. Hvu-MIR159 and its target MYB6 could be excellent candidates for this type of research, as they exhibited the greatest changes under salinity conditions in this study. In addition, studies on the expression of barley miRNAs in response to different environmental stresses could allow the identification of good candidates that could be capable of responding to diverse types of stresses. This could lead to the ability to genetically engineer plants that can adapt to various environmental conditions.

The comparative analysis of miRNAs between bacteria of the genus *Thalassospira* and the barley *Hordeum vulgare* L revealed the homologous relatedness of these two distinct kingdoms. These putative bacterial miRNAs could represent a source of novel genes, together with other salt tolerant genes in halophilic bacteria such as ectoine, trehalose, glycine betaine or Na^+ and H^+ antiporters (Das et al., 2015), that could be used for enhancing agricultural productivity in transgenic plants. Therefore, it would be useful to carry out a transgenic study, using highly expressed bacterial miRNAs in barley. A comparative analysis of miRNA in bacteria and salt responsive miRNAs in plants should be further undertaken through the large-scale miRNA data collection using a wide range of bacterial and plant species. Due to the occurrence of miRNAs in algae (Zhao et al., 2007, Cock et al., 2010, Liang et al., 2010), it would also be useful to determine the sequence relatedness of miRNAs between algae, bacteria and plants; this may uncover important information relating to the evolution of miRNA.

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Appendices

Appendix 1 miRNAs retrieved from bacteria of the genus *Thalassospira* as identified by CID-miRNA analysis.

miRNA*	Location	Sequence
***T. alkalitolerans* JCM 18968^T**		
T. alka_5p_4279	ATWN01000008.1_4279	CAAGGTGGTAAGCGGTGGTTAT
T. alka_3p_4279		ATAACCGCCGCTTTCTCCTTTC
T. alka_5p_3838	ATWN01000007.1_3838	CGAAACGGATCACCGTTTCAGG
T. alka_3p_3838		AGGGCCTTTGAATAATGGTCGG
T. alka_5p_329	ATWN01000001.1_329	GGATTAGGAGGCTGGCAACAAC
T. alka_3p_329		TGCCAGCCTCCTTTTTAGGTTC
T. alka_5p _582	ATWN01000001.1_582	CAAAATTCATCATGTTCGACCT
T. alka_3p _582		CGGGGTCGATGATTTGCTTTTT
T. alka_5p_1519	ATWN01000002.1_1519	AAGGGTTGGTTCCGACCTATCT
T. alka_3p _1519		TATCTTGAACAGTCGATGCAGG
T. alka_5p_333	ATWN01000001.1_333	TTGTTACTTCAATGATCCGGTT
T. alka_3p _333		TTCGGGGCTGGATCGTGTGCTG
T. alka_5p_3811	ATWN01000007.1_3811	GCTGGTATCGGCCTTTGCCGGG
T. alka_3p_3811		CCGGGTTTGACCGGATGAAGGC
T. alka_5p_3149	ATWN01000005.1_3149	TCATCCTGCTTGAAGCGCACCG
T. alka_3p_3149		GGCGCTTCGGGCCGGAATGGCG
T. alka_5p_4942	ATWN01000010.1_4942	TTAATCCGGACCCATTAATTAT
T. alka_3p_4942		CATAATTAATGTGTTCGGAACT
T. alka_5p_2354	ATWN01000004.1_2354	CAGTGTGTTCGAATGATCCGGT
T. alka_3p_2354		CGATTGATCTGTTTGAAGACAT
T. alka_5p_4120	ATWN01000007.1_4120	CCTAATCAGCAGTTATTTCCTG
T. alka_3p_4120		AAAACCCCGCCAGAATGTTCTG
T. alka_5p_2265	ATWN01000004.1_2265	AGTTGCGCATCGATATGGAAGA
T. alka_3p _2265		TTGAGCTTCTGATACCGTATGC
T. alka_5p_5955	ATWN01000015.1_5955	CAAAACGCCTGCTGATTGACTG
T. alka_3p _5955		TCATTCGCTGCGTAAACTTGGC
T. alka_5p_1023	ATWN01000002.1_1023	TTTATCTGTGTCGGGTGTGGAA
T. alka_3p_1023		GACATCACACCGATGGAAAATC
T. alka_5p_5176	ATWN01000011.1_5176	TTTGTTTTTGATACAAGCTAAA
T. alka_3p_5176		TTGCATTAAGAACGGGGTCACG
T. alka_5p_903	ATWN01000001.1_903	CGTTCGCTCTGCATGCAGCCGA

miRNA*	Location	Sequence
T. alka_3p_903		TGTTTGATGAGCCGACGTCCGC
T. alka_5p_3591	ATWN01000006.1_3591	AGATGTCCGCATCAGCGCCCTT
T. alka_3p _3591		CGCCCTTGGCAAGAAAGGCCGT
T. alka_5p_1819	ATWN01000003.1_1819	ATCAGGTCGAAGCCATGACCAT
T. alka_3p _1819		ATGACCATGGCCGACAAAATCG
T. alka_5p_5116	ATWN01000010.1_5116	TTGAAAGATATATATCTTCCTG
T. alka_3p _5116		CCGCACAAATCCGGTCAACCAT
T. alka_5p_2932	ATWN01000005.1_2932	AGGCGGCAAACTTGCCGCCTCA
T. alka_3p_2932		CTCATTTTTTGATTGTTGTATT
T. alka_5p_4684	ATWN01000009.1_4684	TCTGGCATCGGCGTTTCTATCG
T. alka_3p_4684		CGTCGTCTGATCCGCTTTGCCA
***T. mesophila* JCM 18969^{T}**		
T. meso_5p_159	ATWN01000001.1_159	CCGGGCTGTTATCCAACTCCGG
T. meso_3p_159		TACAATTGGCGTTAACCCCGCT
***T. povalilytica* Zumi 95^{T}**		
T. pova_5p_2290	AMRN01000009.1_2290	TCCGGGTCGGGTAAATCGGTTT
T. pova_3p_2290		GGTCTGATTGATTTTCCCGGTC
T. pova_5p_489	AMRN01000001.1_489	TTCATCCAGCCGATGCGCGCGA
T. pova_3p_489		CGCGCGACACAGTTAAAGATCC
***T. profundimaris* WP0211^{T}**		
T. profu_5p_28449	AMRN01000004.1_28449	TTATGCGAAATTGAGAAGCGTT
T. profu_3p_28449		ATTTGTTTCAGGCATAGGACAT
T. profu_5p_49903	AMRN01000010.1_49903	CGAGGCCCCGATGGGCCTCCCC
T. profu_3p_49903		CTCCCCAGATGGGGAAAGCCCC
T. profu_5p_52536	AMRN01000011.1_52536	AAAAAAACGCCTGACCGGTTTT
T. profu_3p_52536		TCAGGCGTTTTTTTGTTCGTGT
T. profu_5p_56451	AMRN01000014.1_56451	AAAGCTTCCTCTTTGAAAATCT
T. profu_3p_56451		TATCTTTTTCAAAGGAAGTCAT
T. profu_5p_37885	AMRN01000006.1_37885	AGCGACAACGCCGGTGGGATCA
T. profu_3p_37885		TGCCACCGGCGTTGTTGTCTTC
T. profu_5p_32508	AMRN01000005.1_32508	ACCCCCGGTCGTTAGGCCGGGG
T. profu_3p_32508		GGGTGTTTTTTTGCTATAGTTC
T. profu_5p_14495	AMRN01000002.1_14495	AAGAAGCAGCGTCGGCCAGCCA
T. profu_3p_14495		GCCGGCGCTGCCTCAACTCGTT
T. profu_5p_40748	AMRN01000007.1_40748	TCGGTTGGTAAAATCGCCGATA
T. profu_3p_40748		CGATATCTATGCCCACCGTGGG
T. profu_5p_57479	AMRN01000017.1_57479	TACCTCGATCCATATCGAGGAA

miRNA*	Location	Sequence
T. profu_3p_57479		AGGAATTCGAAGTCATGGCCCG
T. profu_5p_5865	AMRN01000001.1_5865	TGGATTAAAAAAAAGCGCCGCC
T. profu_3p_5865		CGGCGCTTTTTTTTGTTGGCTG
T. profu_5p_24901	AMRN01000003.1_24901	CCGAAGAGGCCCAGGTGCGGTT
T. profu_3p_24901		ATTCCCGCCTGACGATCTTTGT
T. profu_5p_17082	AMRN01000002.1_17082	CGGGGGTGTCAGCTTTGCCGGG
T. profu_3p_17082		TTCGGACCTGATGGTCCCGCCA
T. profu_5p_9602	AMRN01000001.1_9602	ATCAAAAAGGCGGAGCTGATTT
T. profu_3p_9602		CTCCGCCTTTTTTTTGTTCGAG
T. profu_5p_49438	AMRN01000010.1_49438	AATCATCGATCCGTTGATCTTC
T. profu_3p_49438		AACTTTGTGCAGCTGTTTGGTC
T. profu_5p_33610	AMRN01000005.1_33610	CTGAAAACCGAAAAAGGTCCAT
T. profu_3p_33610		CATTTCCGGATCTTTTTTCTGT
T. profu_5p_56952	AMRN01000015.1_56952	AATTTACCCCTGTCCCGTCCAA
T. profu_3p_56952		ATGAGATAGGGGCTGATCTCGA
T. profu_5p_45214	AMRN01000008.1_45214	CGCGGCGGTGGCGTTGCCGAAC
T. profu_3p_45214		AACGTGATGGCGTCATGCACCG
T. profu_5p_20856	AMRN01000003.1_20856	TCGATCAATATCTACAGCCAGA
T. profu_3p_20856		TGATGTTGGTCGGGCTGATGGC
T. profu_5p_3073	AMRN01000001.1_3073	TTCTGTTTTTGGATCGGCCTGA
T. profu_3p_3073		ATCGGCCTGATTGCTGTTATGG
T. profu_5p_29989	AMRN01000004.1_29989	CGGTTGCAATTGCGACCACCAC
T. profu_3p_29989		CCTTATAGCGGAATGCGCCCTG
T. profu_5p_17078	AMRN01000002.1_17078	CTCCAGCTGTCGGAGACAGGGC
T. profu_3p_17078		TGCCTCCGAACAGTCTGGGAGA
T. profu_5p_43277	AMRN01000008.1_43277	CCGGGAAGGATATTCTTCCCGG
T. profu_3p_43277		CGGCCTTTTTGATGTGTTGATG
T. profu_5p_41148	AMRN01000007.1_41148	AACAAAACCCGCAAGGCCAATG
T. profu_3p_41148		TTGCGGGTTTTGCTGTGATGTT
T. profu_5p_54785	AMRN01000013.1_54785	GGGGGGAAAAGTTCCCTTGCCG
T. profu_3p_54785		TTGCCGAACGGCTGAAAGAGCT
T. profu_5p_7550	AMRN01000001.1_7550	GTGATCTTGAGCTTGATCACGG
T. profu_3p_7550		AAGGTCAATGTCCATGGCGGGG
T. profu_5p_17011	RN01000002.1_17011	TATGATGATGCGCGACAATTTT
T. profu_3p_17011		TAGTTCATAATCATAACGGCGA
T. profu_5p_33954	AMRN01000005.1_33954	GAAAACGATGGGGAGTGGGCGC
T. profu_3p_33954		GTCCACTTCCGCTGCGCCGTTT

miRNA*	Location	Sequence
T. profu_5p_44623	AMRN01000008.1_44623	TGAATCCAATGCAAGTGATGGT
T. profu_3p_44623		ACCATAATTTTGCTGGGCTTGG
T. profu_5p_29018	AMRN01000004.1_29018	CACCGGAACGGCATCCCGGTGC
T. profu_3p_29018		CGGTGCGTTTGTTGGTTTGGCG
T. profu_5p_43344	AMRN01000008.1_43344	ATCTGTTTTGGGGCGATCCGAA
T. profu_3p_43344		CGAACTTTGTTCCTGCCATGAC
T. profu_5p_30109	AMRN01000004.1_30109	CAATCTGTTGCAGTGCCTGATC
T. profu_3p_30109		CTGATCTGCTTCGTTACGGATA
T. profu_5p_7879	AMRN01000001.1_7879	TGCCTATCGCGTCGACGAGGTG
T. profu_3p_7879		TGTCGAGGCGGCTGGTCTGCGT
T. profu_5p_43079	AMRN01000008.1_43079	AAGAACACCGTTTGATCATCGT
T. profu_3p_43079		CGAAGACATGAAAACGCTGTTG
T. profu_5p_778	AMRN01000001.1_778	CACCGATGTCGAAAGATCTTCG
T. profu_3p_778		TTCCATATCGTGTCGTTATTCA
T. profu_5p_28659	AMRN01000004.1_28659	CGATTTCAATCTCGATGAAGTT
T. profu_3p_28659		AAGTTTTATTTTGATAGCGCAG
T. profu_5p_57432	AMRN01000017.1_57432	ACTACTTCACCGGTCCGACCGC
T. profu_3p_57432		CTGATCCGGCTTCGGTCGCAAA
T. profu_5p_11482	AMRN01000001.1_11482	TTTGGCGGTTTCTCGCCCGAGG
T. profu_3p_11482		CGAGGCATTGGCTGGCCGTGTC
T. profu_5p_14407	AMRN01000002.1_14407	TTGCGTCCGTTTATTGGCGAGG
T. profu_3p_14407		AGCGTGTCATGGCCTTCATGCC
T. profu_5p_32260	AMRN01000005.1_32260	AATTGTATGTGCAATAATGCGA
T. profu_3p_32260		GCGTTCGGAGGATTGCACATGC
T. profu_5p_3800	AMRN01000001.1_3800	ACCCGATGCTTACGCCGGACCC
T. profu_3p_3800		CTCATGAGTAATGTGTTCGGAA
T. profu_5p_38494	AMRN01000007.1_38494	TTCAATCTTGCCCGCTATCTTG
T. profu_3p_38494		ACATGTGGTCAAGGTTGTTCGA
T. profu_5p_51013	AMRN01000010.1_51013	CAGCATGCTGAATGCGGCGCGC
T. profu_3p_51013		ATCGCTTTCGGCTTGGTGTAGT
T. profu_5p_12371	AMRN01000002.1_12371	AATGGGTTGATGTCGGGGCACG
T. profu_3p_12371		AATAGCCCACGGCATCACTCAT
T. profu_5p_33977	AMRN01000005.1_33977	CATCAGAGATGCAGGGCCTATT
T. profu_3p_33977		CAGGGCCTATTTAATTGGTAGC
T. profu_5p_45949	AMRN01000008.1_45949	CTGGGCCAACGGGCTTGTCATT
T. profu_3p_45949		GTCATTCTGATTGCCTGCATGA
T. profu_5p_19048	AMRN01000008.1_45949	TGTGATGGTTTCTTCTATCGCA

miRNA*	Location	Sequence
T. profu_3p_19048		GTCGGTGGCGGTAACACCGCGG
T. profu_5p_38661	AMRN01000007.1_38661	TTTCAACAACGCCCGTTGATTG
T. profu_3p_38661		ATTGAAATCCCCCGCCTAAACC
T. profu_5p_42425	AMRN01000007.1_42425	ATTTTGTACCTGATGAAACGGC
T. profu_3p_42425		CGTTTTGTTAGGTGTTAACCTG
T. profu_5p_55792	AMRN01000014.1_55792	TCGTGCCGATGGCTCGGCGATC
T. profu_3p_55792		ACGGCGAGCCGATCGGCACGCG
T. profu_5p_15172	AMRN01000002.1_15172	ATTTTGCAATCGCTTATCGCTT
T. profu_3p_15172		AGCGATCCGCGATTTGTTGAGC
T. profu_5p_54600	AMRN01000012.1_54600	TTTCCACTGCTGGAAACGCGGC
T. profu_3p_54600		ACGCGGCGGTCATGGCTGGATG
T. profu_5p_18009	AMRN01000002.1_18009	GTTTGGCGACCCTGATCGACCG
T. profu_3p_18009		ACCGGTCCATCTCGGCCGCCGA
T. profu_5p_44551	AMRN01000008.1_44551	CAGGGCATTTCTGCCCTAGCCT
T. profu_3p_44551		CTAGCCTTTCGGATGGTTTGCG
T. profu_5p_24235	AMRN01000003.1_24235	TATGCCAACAATCCGACCGGGT
T. profu_3p_24235		GCGGTCTGGATGTTGGCCTGCC
T. profu_5p_44441	AMRN01000008.1_44441	AGTGTACTCAGTGAGTATGCTC
T. profu_3p_44441		ATGCTCATTTAAATCGGAGGCG
T. profu_5p_18810	AMRN01000002.1_18810	CGGCGATCATGATTGCCGCGGC
T. profu_3p_18810		CGCGGCCGCCGGCGCCTATGTG
T. profu_5p_41532	AMRN01000007.1_41532	TCTCGGGGACGGGTGCGGAACT
T. profu_3p_41532		AAGTCCGCGCAGCCTACCTCGA
T. profu_5p_44043	AMRN01000008.1_44043	GGGGGAATGCGTTCCCTATGCC
T. profu_3p_44043		ATGCCCGGTATAATGAAACGGT
T. profu_5p_21694	AMRN01000003.1_21694	AGCAAAAGCTGCCTAATTAAGG
T. profu_3p_21694		CTTCTGCTTTACAGACAGAATT
T. profu_5p_49945	AMRN01000010.1_49945	TGTCTTTTTCTGACGTTTTTTC
T. profu_3p_49945		CGTTTTTTCTCAAAAAAGGGTT
T. profu_5p_17170	AMRN01000002.1_17170	TTGTCTGTCAAACAGGCAAGGA
T. profu_3p_17170		AAGGATTGCGGTCGGCCTTACT
T. profu_5p_51572	AMRN01000011.1_51572	TGACGCAGAGGCTTTCTCTCAT
T. profu_3p_51572		AGGTGGCCTTTGGATCACCCGG
T. profu_5p_56542	AMRN01000015.1_56542	CGATCGCCGTCACTTCGGCCTT
T. profu_3p_56542		TGCCGAGGGCCCGCGTGCGGTC
T. profu_5p_30378	AMRN01000005.1_30378	CGGATGAATTTTCCGTCCTGGA
T. profu_3p_30378		TCCTGGATGGCCACGAAATAGA

miRNA*	Location	Sequence
T. profu_5p_40629	AMRN01000007.1_40629	TTGTTGTAATACAGAAGCGGGG
T. profu_3p_40629		CAGAAGCGGGGTCGAGCTGTTG
T. profu_5p_12216	AMRN01000002.1_12216	GTCGGCGTTGTCGCGCTGTTCA
T. profu_3p_12216		TTCAAGGAGCCGCTGCATGTTG
T. profu_5p_15457	AMRN01000002.1_15457	CGCAAGGAAAAGCCCCGCAGAC
T. profu_3p_15457		GGGGCTTTTTTGTGTCCAGGAA
T. profu_5p_50983	AMRN01000010.1_50983	TATGGCGTTGCTGTAGTGTTTG
T. profu_3p_50983		TAGTGTTTGCACTGGTGGTTAT
T. profu_5p_5000	AMRN01000001.1_5000	TGGGTAGGTGTTCACCTATCGG
T. profu_3p_5000		GGTAACGAACTGTAATTACACT
T. profu_5p_480	AMRN01000001.1_480	CCCCCCGTCTGTCGTTCTTCTG
T. profu_3p_480		TGGAAATCGCCAAGATGCGCGC
T. profu_5p_14005	AMRN01000002.1_14005	AGGGCCACGCCCAGGTCAATGC
T. profu_3p_14005		CACATGGCCGGATGCAGCCAAC
T. profu_5p_44486	AMRN01000008.1_44486	GGACGCATTGATGCGACCCGAG
T. profu_3p_44486		CCGAGTTCGGGCAGGTGTGCCA
T. profu_5p_1328	AMRN01000001.1_1328	TGATCCTCACAGTGTTGACCGC
T. profu_3p_1328		AATCGCGCTTGGCTTTATTGGC
T. profu_5p_18148	AMRN01000002.1_18148	TGCCGCCGCGACCATCGCGGGG
T. profu_3p_18148		CCCCGACGCTGATCGCGCGGGG
T. profu_5p_12680	AMRN01000002.1_12680	ATCGGGACAAATCGGTGCCGAT
T. profu_3p_12680		ATGGTGGCATTGGCAACCGCAC
T. profu_5p_12074	AMRN01000001.1_12074	ATCGGGGCCGGATCCGGCGGCC
T. profu_3p_12074		CCGGTGCGGTTCAGATGGGCGC
T. profu_5p_53216	AMRN01000012.1_53216	CTCTTTCCGCTGAACTAAGCT
T. profu_3p_53216		CATGTTTCATCGGAGGGACATC
T. profu_5p_24324	AMRN01000003.1_24324	CGTCGGTCGTGAGCTGGCGCGC
T. profu_3p_24324		TGGCGCGCTGGAGCCCGGAACA
***T. xianhensis* P-4^{T}**		
T. xian_5p_16668	CP004388.1_16668	AGACGTGACCTTCGGGTCGCGT
T. xian_3p_16668		CGTCTTTTTTATTGTCTGGTGG
T. xian_5p_6822	CP004388.1_6822	CAATTAAAAACCCCCTCAGGCG
T. xian_3p_6822		AGGGGTTTTTTAATTGGTAGCC
T. xian_5p_20844	CP004388.1_20844	CGCAGCTTTCCGGCTGCGGGGC
T. xian_3p_20844		GGGCTTTTTTACATTTGGTTGC
T. xian_5p_2740	CP004388.1_2740	GAGTCCATGGTGCTGCGAGCTC
T. xian_3p_2740		ATTCCCGCGTGCGCGGGAATGA

miRNA*	Location	Sequence
T. xian_5p_9958	CP004388.1_9958	ATATTTCGTAACCATATTTACG
T. xian_3p_9958		AAATATCGTAGGCCGATCACGA
T. xian_5p_3333	CP004388.1_3333	ATCATCGGTGACGGTGGTGGCT
T. xian_3p_3333		AGCCATCGCTGCCGGTGATCCC
T. xian_5p_15747	CP004388.1_15747	CTTCTTTAACGAACTTGAAGAA
T. xian_3p_15747		AGAATGGCTTTCGGGCTTTACC
T. xian_5p_5035	CP004388.1_5035	CCCGGGACATGTCCCGGGCGAA
T. xian_3p_5035		GGCGAAGTTTTGTGTGCGTATC
T. xian_5p_21040	CP004388.1_21040	AAAACCTCTTGCGCGGTCTTGA
T. xian_3p_21040		CGCGCAAGAGGCTTTTGCATCG
T. xian_5p_3489	CP004388.1_3489	ACGGATCGTTGGAAAACCGTCG
T. xian_3p_3489		AAACCGTCGACCGGGAGCTCGG
T. xian_5p_22881	CP004388.1_22881	CACCATCCTGACCGCCAGCATG
T. xian_3p_22881		TATTTATCGGGTTGGGCGGCGA
T. xian_5p_14589	CP004388.1_14589	TATCCTCAAAGCCGACGACCGA
T. xian_3p_14589		ACTCCCGCTTTCGCCGGAGTGA
T. xian_5p_2738	CP004388.1_2738	GAGTCCACCGGGCTTGCGGGTT
T. xian_3p_2738		ATTCCCGCGTGCGCGGGAATGA
T. xian_5p_12893	CP004388.1_12893	AATGGGCGGGGCAACCCGCGAT
T. xian_3p_12893		GATCTTTTCCATTGGATATCGG
T. xian_5p_22216	CP004388.1_22216	CCCCGTACTTTGTACGGGGCTT
T. xian_3p_22216		GGGCTTCTTTTTTTGCCGGTCG
T. xian_5p_17206	CP004388.1_17206	GGCGTCGGGCCTTGATGGCCGT
T. xian_3p_17206		GCCGCAAGACCGCCGCCGCCAA
T. xian_5p_19488	CP004388.1_19488	TCGGTTTCTGCCTTGGCGGGCT
T. xian_3p_19488		CACGCGAACTGATGGCGATGCC
T. xian_5p_3512	CP004388.1_3512	AATCAGCCTGATTATTCACTTT
T. xian_3p_3512		ATTCACTTTTTGCATTGCGGCA
T. xian_5p_20129	CP004388.1_20129	AAGGGTCGCCCAGACATCGCCA
T. xian_3p_20129		TTGCGGGTAAAACTGGCGATGC
T. xian_5p_6229	CP004388.1_6229	AGGGCATCGGTCAGGCCTTTGG
T. xian_3p_6229		AATGCATGCATGGCAAAACCGA
T. xian_5p_4741	CP004388.1_4741	CTGCCGATTGTGCTGCTGATCC
T. xian_3p_4741		GGGTGATCGTAGCACTCCGGCG
T. xian_5p_17016	CP004388.1_17016	AATGGCCAGCTTTCCCCGATCC
T. xian_3p_17016		ATGCAAGGTTATTGATCGGACC
T. xian_5p_16719	CP004388.1_16719	ATTTTGGATGCCTTGCGTGTTT

miRNA*	Location	Sequence
T. xian_3p_16719		GAAGGAATTCATCATTTTGCCA
T. xian_5p_22099	CP004388.1_22099	TTGCCGGCTGTGATCATCATGT
T. xian_3p_22099		TGGTGTCGGGCCTGCGCGAACA
T. xian_5p_16407	CP004388.1_16407	CATTTTCCGCCTCGCAGACTTT
T. xian_3p_16407		CCTGCGCGATTGCGCTATCGAT
T. xian_5p_18366	CP004388.1_18366	TATAATGCAACCGAAAGCCGTC
T. xian_3p_18366		GGCGGCTTTCGCGTTTTGGGGG
T. xian_5p_4407	CP004388.1_4407	CAACAGTGCGGCCGAGGCGCGT
T. xian_3p_4407		TGCGCAGCACGGTTGCCGATAT
T. xian_5p_17681	CP004388.1_17681	CTATTCCATCGGTTACATGTCG
T. xian_3p_17681		CATGTCGCACGATCCCGACAAG
T. xian_5p_23642	CP004388.1_23642	TCTGGGCCTGCCGACGGTTCTT
T. xian_3p_23642		CATTGTTCTCGTAGGCCCGCCA
T. xian_5p_3091	CP004388.1_3091	ATCGGCGGCAGTTTTGGCGTCC
T. xian_3p_3091		CCAAAGATGCTGCCGTCGCCCA
T. xian_5p_22837	CP004388.1_22837	ATTGACCTTGGTTTCCAGCGTG
T. xian_3p_22837		CAGCGTGTGATCGAAATCTGGT
T. xian_5p_17518	CP004388.1_17518	AGCTGTGCCGCGTCATGTTGCC
T. xian_3p_17518		AACGGGCGCTGGGCAAAGTTGC
***T. lucentensis* QMT2^{T}**		
T. luce_5p_167793	ATWN01000002.1_ 167793	TATCCACTAGACATCATTTGGA
T. luce_3p_167793		TTGGATAATTATATAACAATTT
T. luce_5p_124614	ATWN01000002.1 _124614	TAAAGAACAAAAGCTCCGCAGG
T. luce_3p_124614		GAGCTTTGTTTTTTGCGTAAAA
T. luce_5p_247382	ATWN01000003.1_247382	CCAACCGGGTGTTTAACCCGCA
T. luce_3p_247382		AGTATGGGCAGGCTCAATCGCG
T. luce_5p_523350	ATWN01000010.1_523350	ATGTCCATGACCGTGGTGGTGA
T. luce_3p_523350		TGACCATGACCATGACCATGAC
T. luce_5p_637434	ATWN01000020.1_637434	TTTTGTGGCACCTGACAACTTT
T. luce_3p_637434		CGAGTGCCACAAAAGATGACAT
T. luce_5p_312757	ATWN01000005.1_312757	AACCAACCATTATCGGTAAACA
T. luce_3p_312757		TTTTACCGTAATGGTTGGTTTT
T. luce_5p_608218	ATWN01000013.1_608218	TGGTCTTTTCAAACCAGCTCGA
T. luce_3p_608218		TCGATGCCGGTGGGCTTGACCG
T. luce_5p_496507	ATWN01000009.1_496507	AGATTTTCAAAAAAGGCGGTGT
T. luce_3p_496507		CACCGCTTTTTTTATATCCGGA
T. luce_5p_269288	ATWN01000004.1_269288	CATTGATGCATCAATGATACGT

miRNA*	Location	Sequence
T. luce_3p_269288		TCAATGATGCATTCGAGTATCA
T. luce_5p_559608	ATWN01000011.1_559608	ATATGCGATGATCTATGGCTGC
T. luce_3p_559608		TTTGGCGTTGCCTACGCCTATT
T. luce_5p_170616	ATWN01000002.1_170616	CCCGGCCCTTGCCTATGCTGGC
T. luce_3p_170616		CTTTGCAATTGCCAAGAAGGAA
T. luce_5p_158773	ATWN01000002.1_158773	AAACTGCCAGCTCGTGCCGTTT
T. luce_3p_158773		TTTCGGTCCGGATGATGCCGGA
T. luce_5p_123341	ATWN01000002.1_12334	CAGCCCAAGCCCGCGCTTGGGC
T. luce_3p_123341		GGGCTGTTTTTCTTCAATCAGG
T. luce_5p_528656	ATWN01000010.1_528656	TTCGGTGCTCACGTACTTTTAG
T. luce_3p_528656		TGCGCTCCGATGCGCGTGAACC
T. luce_5p_542101	ATWN01000010.1_542101	TGTCCTGTGGACACTCATATTG
T. luce_3p_542101		AATATGAGTGTCCACAGGCTCT
T. luce_5p_39407	ATWN01000001.1_39407	CCGGAACGGTCCAGTTGTTCCG
T. luce_3p_39407		CGGCCTTTCAGGGTGCCTTCGC
T. luce_5p_185415	ATWN01000002.1_185415	AAGTTTCTTCCCACCCTTTTGA
T. luce_3p_185415		AGGAAGAAATATCCCGCCTAAC
T. luce_5p_248131	ATWN01000003.1_248131	ACGATTTTGTCGGCCATGGTCA
T. luce_3p_248131		TGATCATGGGTGACATAGATCA
T. luce_5p_429076	ATWN01000007.1_429076	AAGCCCGCCGGTTTGGCGGGCT
T. luce_3p_429076		CGGGCTTTTGTCTGGCAACATA
T. luce_5p_228121	ATWN01000003.1_228121	AATAAAAGACCCTGCCACCGTG
T. luce_3p_228121		AGGGTCTTTTTATTGGTTGCGG
T. luce_5p_543369	ATWN01000011.1_543369	AAGGCCGCAGCATGATGCTGCG
T. luce_3p_543369		TGCGGCCTTTGTCATTCACATC
T. luce_5p_302533	ATWN01000004.1_302533	CCGCCTTCGCGGGGATGACGGT
T. luce_3p_302533		TCATTCCCGCGCAGGCGGGAAT
T. luce_5p_498549	ATWN01000009.1_498549	ATTCAAGCTGGCTGATCATTGG
T. luce_3p_498549		CGTCAGTTTCAACCACATGCTT
T. luce_5p_48057	ATWN01000001.1_48057	CACGGATTTCTCTGTCGATATG
T. luce_3p_48057		TGTCGATATGGAAACGCTTAAG
T. luce_5p_528636	ATWN01000010.1_528636	TTCGGTGCTCACGTACTTTTAG
T. luce_3p_528636		TGCGCTCCGATGCGCGTGAACC
T. luce_5p_525349	ATWN01000010.1_525349	AAGAGCCTGTGGACACTCATAT
T. luce_3p_525349		CAATATGAGTGTCCACAGGACA
T. luce_5p_285535	ATWN01000004.1_285535	TTTGAGGAATTCCTCGATCAGC
T. luce_3p_285535		ATCAGCGGGAAATAATGAATTG

miRNA*	Location	Sequence
T. luce_5p_13919	ATWN01000001.1_13919	AAAGGACGAAGTGTCCGGTCCG
T. luce_3p_13919		GGTCCGATTACCTTACCGCCAT
T. luce_5p_389406	ATWN01000006.1_389406	TCGTGCGTCAGCTTGGCGTGAC
T. luce_3p_389406		TCACCCGACCTGACCATGGTCG
T. luce_5p_307016	ATWN01000004.1_307016	AACTTGCCGGGGCTAATACGGT
T. luce_3p_307016		AATTACCGTATCCGCCCCGGCG
T. luce_5p_471094	ATWN01000008.1_471094	TTGCCAAATTCAAAAGGCGGTG
T. luce_3p_471094		CGCCGCCTTTTTTGTGTGCATA
T. luce_5p_311628	ATWN01000005.1_311628	ACCCGCATGAAATGCCGGTCGT
T. luce_3p_311628		CCGGTCGTCTTCTGTAAACGCG
T. luce_5p_384973	ATWN01000006.1_384973	TCATCGCGCACAAAGCCATTGT
T. luce_3p_384973		TGTCGTTTTGTGCCGATAACGA
T. luce_5p_7447	ATWN01000001.1_7447	GCGGTGGCATGGTCACCAACGC
T. luce_3p_7447		AACGCATCAGAAAGGCGTTGAT
T. luce_5p_245911	ATWN01000003.1_245911	TGGGGCCAAATGTGATCTTGGT
T. luce_3p_245911		CATGGGCGCGTTTGGCCTGTGC
T. luce_5p_879	ATWN01000001.1_879	ATGCCAAAAGAAAAACACCCCG
T. luce_3p_879		AGCGGGGTGTTTTGTTAAGCTT
T. luce_5p_464921	ATWN01000008.1_464921	TGTGGCCGTTTTTGGTCATCAC
T. luce_3p_464921		GATGACAATTACGGTCGGGATT
T. luce_5p_171055	ATWN01000002.1_171055	CCAGCTTCCTGCCGCCAACAGA
T. luce_3p_171055		AACAGATCAAGTGACGGTGCTT
T. luce_5p_198770	ATWN01000003.1_198770	TGACTGGCGAATATGACGTCAT
T. luce_3p_198770		CATGTTGCCGTTTCGCGATGGT
T. luce_5p_350700	ATWN01000005.1_350700	ACGTGACATGATGCCGGGCCGA
T. luce_3p_350700		TTCGGCCCGGTTTGCATTTGTT
T. luce_5p_82704	ATWN01000001.1_82704	AATATCGCAAGGGCGTTCTGGG
T. luce_3p_82704		TGCCATCGAAGCCCTTTCGGTG
T. luce_5p_273434	ATWN01000004.1_273434	CAGGTCAAAAAAAGCGCAACGG
T. luce_3p_273434		GGTTGCGCTAGTTTTTGGCCGT
T. luce_5p_273440	ATWN01000004.1_273440	AAATCAAAATTACTTAATTTTA
T. luce_3p_273440		TTTATTTTCGATTATTTTTATG
T. luce_5p_142582	ATWN01000002.1_142582	GCTCTGCAAGACGTTGCTGACA
T. luce_3p_142582		TGACATGAATGGTGGCCCGGTG
T. luce_5p_449960	ATWN01000008.1_449960	TATGGGCTTTATCGCCTGCACA
T. luce_3p_449960		AAATGTTTGTCGCCTTTGCCCG
T. luce_5p_437928	ATWN01000007.1_437928	TAAAAAAGGGCGGCCTGTCATA

miRNA*	Location	Sequence
T. luce_3p_437928		AGGTCGCCCTTTTTCGTTAACT
T. luce_5p_127263	ATWN01000002.1_127263	TGTCGGGTTGAACGACAGCGCA
T. luce_3p_127263		CGCACGATGCCATTGGAAGCGG
T. luce_5p_210055	ATWN01000003.1_210055	TCGGCGGATTTTTGACCCGATT
T. luce_3p_210055		ATTTAAAGTCCGGTTCGACCGC
T. luce_5p_432525	ATWN01000007.1_432525	CGCGCATTCCTCAATGCGGTGT
T. luce_3p_432525		TGCGGTGTCAAACGGGGTGCTG
T. luce_5p_112640	ATWN01000002.1_112640	ATCGGCACCAATTTTGCCCTTT
T. luce_3p_112640		CGGCAAAACTGGTGCCGATTAA
T. luce_5p_529459	ATWN01000010.1_529459	CGGTGCAAAAGTCGCCGCTATA
T. luce_3p_529459		CGCTATAGCTGCTTTGCCAGTT
T. luce_5p_48943	ATWN01000001.1_48943	TTTGCGGCGTCCTTTCAATCGT
T. luce_3p_48943		CGCCGCCTTTTTCGTGGTTGTC
T. luce_5p_294505	ATWN01000004.1_294505	AACGGCCTATTTGGGATGAAGC
T. luce_3p_294505		GGTCTCAAACGGCCCGGCAATT
T. luce_5p_485577	ATWN01000009.1_485577	CCCGGTCTGGAATCGGTTGGCG
T. luce_3p_485577		GGTTGGCGATCGTACGTATGTG
T. luce_5p_475755	ATWN01000008.1_475755	AGCTTGGCAGGAATGAATGGTG
T. luce_3p_475755		CCACTCATTTTCTTGCAAAGAT
T. luce_5p_601689	ATWN01000013.1_601689	CGCCCCGCAGATTTGCGGGGCG
T. luce_3p_601689		GGGGCGTTTTTTTGTGCCGGTC
T. luce_5p_385818	ATWN01000006.1_385818	CAAAGGATTGCTTGGCCGCCCC
T. luce_3p_385818		TTGGCCGCCCCGAAAGCTACCT
T. luce_5p_338996	ATWN01000005.1_338996	TGATCCAGACCGGCGAGGTCGG
T. luce_3p_338996		CCTGTTACTCGCCGGTCTTTTT
T. luce_5p_17121	ATWN01000001.1_17121	AGTGCTGCATCGCTCAGGCGCA
T. luce_3p_17121		TGCGCGACGTTGCAGCACAAGT
T. luce_5p_56287	ATWN01000001.1_56287	TTGCGGTTCTGTCGAACTCGCC
T. luce_3p_56287		AACTCGCCAGAACGGTTTTTAA
T. luce_5p_218949	ATWN01000003.1_218949	ATCAGGTCGAAGCCATGACCAT
T. luce_3p_218949		ACCATGGCCGACAAAATCGTCG
T. luce_5p_314181	ATWN01000005.1_314181	TGGATGTTATGGATGGGCGGCG
T. luce_3p_314181		CATCTATCTGAAGTTAAGTAAG
T. luce_5p_441886	ATWN01000007.1_441886	AATATGGATCGGCAGTGGTTCG
T. luce_3p_441886		TTAGAACCACTTCAAGCAATCC
T. luce_5p_441217	ATWN01000007.1_441217	TTTACACCGGCAAGGTCGGCTT
T. luce_3p_441217		AACGCTGGGTGGTTGTCGCCCC

miRNA*	Location	Sequence
T. luce_5p_156594	ATWN01000002.1_156594	GTTATTGCAGGAATAGGCAATG
T. luce_3p_156594		AATGCATGATCCTGTGTAACGA
T. luce_5p_260951	ATWN01000004.1_260951	ATTGCTTTACAGCAGCCCGATC
T. luce_3p_260951		CCCGATCATCTGCTTGGGGCGA
T. luce_5p_641508	ATWN01000022.1_641508	AAGCAAAAAGACTGGGGCCGGA
T. luce_3p_641508		ATCCGCCCACGGGCTTGTTGCC
T. luce_5p_109433	ATWN01000001.1_109433	ACTTGCCACATCCTGACGGCCC
T. luce_3p_109433		AGTACGGTAAATTCACCGTGCT
T. luce_5p_556182	ATWN01000011.1_556182	TCGGGTCAGGAGCGGATCGGGG
T. luce_3p_556182		GGGATTTTGGCCCGGCATCGCC
T. luce_5p_399471	ATWN01000006.1_399471	CAACTGTCATCGCGTATCCGGT
T. luce_3p_399471		TTGCTGGTTGATTTTATGCATG
T. luce_5p_492342	ATWN01000009.1_492342	ACCTTACGGTTTAAGCAGGCGT
T. luce_3p_492342		AAGCAGGCGTTTGAAATGTCAT
T. luce_5p_413359	ATWN01000007.1_413359	ATCGGTGTTGCAACTGCCGCCG
T. luce_3p_413359		CGCCGCCGGGATCATTGTGGGC
T. luce_5p_520914	ATWN01000010.1_520914	TTAATCCGGACCCATTAATTAT
T. luce_3p_520914		CATAATTAATGTGTTCGGAACT
T. luce_5p_23446	ATWN01000001.1_23446	CCAGTGTGTGGGGCGGCAGTTC
T. luce_3p_23446		CAGTTCAGATGAGGAAAGGTGG
T. luce_5p_609984	ATWN01000014.1_609984	GTGATGATTTTTTCTTCACCGC
T. luce_3p_609984		AGGTCGATCGCAACGCGTGCTT
T. luce_5p_608572	ATWN01000013.1_608572	ATCGCGTCCCAACGCCGGACGC
T. luce_3p_608572		CGCATTTCAAGGGCCAAGGGCA
T. luce_5p_558336	ATWN01000011.1_558336	TTGATGACGATATTGACGTTCA
T. luce_3p_558336		AATTTAGTCTTTCGACGATCAG
T. luce_5p_160251	ATWN01000002.1_160251	TAATGACAGCATTACCATCATC
T. luce_3p_160251		CATCATCGTCAATGCCGATCTT
T. luce_5p_31255	ATWN01000001.1_31255	TTTCCTGACTTTTGGCGGTCGA
T. luce_3p_31255		GGTTTGCCAATGGGCAGAAACC
T. luce_5p_43818	ATWN01000001.1_43818	AGAATTATCTATTGCGCAAGTG
T. luce_3p_43818		TGCGCAAGTGCGAGATAATTGC
T. luce_5p_66984	ATWN01000001.1_66984	CTCGGCCTGCGCCTTGCGGCCG
T. luce_3p_66984		CCGAGGTTTTCGCGGATCGCGA
T. luce_5p_678	ATWN01000001.1_678	ATCATGTTTTCAAGGGTTTCCG
T. luce_3p_678		CAGTACATGTTCGGTATCGATG
T. luce_5p_533917	ATWN01000010.1_533917	CCCCGCTATATGCGGGGCTGAG

miRNA*	Location	Sequence
T. luce_3p_533917		GATTTTGCGTGCCCGCGCACGC
T. luce_5p_262588	ATWN01000004.1_262588	CCGGCACAGTGTTTGCTGGATG
T. luce_3p_262588		CTGGATGGAAAGTCAGAAATCC
T. luce_5p_246051	ATWN01000003.1_246051	ATCAGGTCATGCAATTTGGCGA
T. luce_3p_246051		ACTGTCGCACCATCGTTTTGAC
T. luce_5p_65195	ATWN01000001.1_65195	TTCCTTCTGGCATGGAGCGGAA
T. luce_5p_65195		AGCGGAACCGTAGAACCGGCAA
T. luce_5p_531055	ATWN01000010.1_531055	TTGCGCTATCGATTCGCAACTG
T. luce_3p_531055		AACTGGCGGGCGCGGGTGAGGA
T. luce_5p_465632	ATWN01000008.1_465632	AATCGGAAGCGACAAACGGCGC
T. luce_3p_465632		AACGGCGCGTCCCAAGGGGCGC
T. luce_5p_458430	ATWN01000008.1_458430	CACGTGGCTTTGATGGTGCGGC
T. luce_3p_458430		ATGAACTATCAGTTCCACGACA
T. luce_5p_22792	ATWN01000001.1_22792	CGATTGTGTTCTGCTTGCGTCG
T. luce_3p_22792		TGCGTCGTCATGTGATGCGGTT
T. luce_5p_282136	ATWN01000004.1_282136	TGCCGGTATTTCGGCCGATGAC
T. luce_3p_282136		GATGACCTGAAGGTCTTCAAAT
T. luce_5p_43186	ATWN01000001.1_43186	CCGTGAAGTAGGCATTCAGAAT
T. luce_3p_43186		CGGGTCTGCTTTCTCACTCATG
T. luce_5p_637355	ATWN01000019.1_637355	GGTTTAATACCGGAATCTGATG
T. luce_3p_637355		CGGAATCTGATGCCGGTTTAAT
T. luce_5p_273060	ATWN01000004.1_273060	GGGTGGCGATTTTGGCCGGTGT
T. luce_3p_273060		AATTGCCAAGCTCGGCCACCTG
T. luce_5p_21030	ATWN01000001.1_21030	TGTGCGAGGCAACACGTTGCGA
T. luce_3p_21030		CGTTGCGATGAACAAAGCTTGA
T. luce_5p_617474	ATWN01000014.1_617474	TGATTACAACGGCTGCACGGCA
T. luce_3p_617474		AAGTTGATTTCGAAATTGATTT
T. luce_5p_394944	ATWN01000006.1_394944	CCCAGCATGATCCGTTCGGCGG
T. luce_3p_394944		GGTGACGGTTGTTGTTGATCCG
T. luce_5p_284817	ATWN01000004.1_284817	GGCACGCTTTACATGTGCCTGG
T. luce_3p_284817		TTGATCTGCGCGCGGCCCTTCG
T. luce_5p_252989	ATWN01000003.1_252989	AGATTGATCTCGACGATGCGTT
T. luce_3p_252989		CGATGCGTTCGACACCGCCAGC
T. luce_5p_252989	ATWN01000003.1_252989	TTGTTACTGACCAGAAAGAGGG
T. luce_3p_252989		AAGAGGGCGGTGTCACCGTTGT
T. luce_5p_38961	ATWN01000001.1_38961	CAGACCATTTTGGTTCTGCCAG
T. luce_3p_38961		AATCGGCGGTATCACCGTTATC

miRNA*	Location	Sequence
T. luce_5p_585162	ATWN01000012.1_585162	GTGATGATCAGCATAGTATCAT
T. luce_3p_585162		ATCATCATACAATAGGATAAGT
T. luce_5p_389408	ATWN01000006.1_389408	TCGTGCGTCAGCTTGGCGTGAC
T. luce_3p_389408		TCACCCGACCTGACCATGGTCG
T. luce_5p_474240	ATWN01000008.1_474240	GAAGTTAAGTGAAACCCCCATC
T. luce_3p_474240		TGGGGGTTTTGCTTTAGGGGCG
T. luce_5p_421879	ATWN01000007.1_421879	AAAGCCTAAAAAGAAACAAGCC
T. luce_3p_421879		AACTGGCTTGTTTTTATAGGTT
T. luce_5p_442710	ATWN01000007.1_442710	CGGATCGGTCACCTGCGCCATA
T. luce_3p_442710		CCATACGTGCACGGGTGTCGTC
T. luce_5p_346590	ATWN01000005.1_346590	CGGGCTTATTTCATGAGTCCGG
T. luce_3p_346590		TCCGGCCCTTGTTTTTTTAGAT
T. luce_5p_544598	ATWN01000011.1_544598	AAAAGAAACCGCCGCCCGGCTT
T. luce_3p_544598		TGGCGTTTTCTTTTGTGTCCTG
T. luce_5p_106175	ATWN01000001.1_106175	GAATAATCTGGCTGATTATTCA
T. luce_3p_106175		ATTCACTTTTAGTTGCGATCCG
T. luce_5p_406605	ATWN01000007.1_406605	CGAAATTTCGATGATGTGGCGG
T. luce_3p_406605		CAGTTCGACCGACATTTTGGCG
T. luce_5p_58702	ATWN01000001.1_58702	GTGATGTGTCTATTCAGGAATT
T. luce_3p_58702		ACGTCCTGATTCAGTTGCAGCG
T. luce_5p_422620	ATWN01000007.1_422620	GACGGCCTTGATGAGGCCGACG
T. luce_3p_422620		GACGATCCGGGCGACATCCTTG
T. luce_5p_5460	ATWN01000001.1_5460	CTTCGCAATCGAAATGCGTATC
T. luce_3p_5460		TGCGTATCAACTGGTCGGCCTT
T. luce_5p_316125	ATWN01000005.1_316125	GATGTGGAGAACCTGTTGGTCG
T. luce_3p_316125		CCCAGCTTTCCCTGTCCCCGGA
T. luce_5p_546117	ATWN01000011.1_546117	AACGCCTTTATCGGCGCACGTT
T. luce_3p_546117		AAGGTGTTATCGTCGAAGAAGG
T. luce_5p_273558	ATWN01000004.1_273558	AATCGTGTACGATTTAAATTAA
T. luce_3p_273558		AAATTAATCGGCTTTGAGTCGT
T. luce_5p_399890	ATWN01000006.1_399890	ATAAACCGGCCCAATCGGATGC
T. luce_3p_399890		TGTGTTGGTTTGTGTGTGACCC
T. luce_5p_242930	ATWN01000003.1_242930	ATATGACGGAAATCGGGAAGTC
T. luce_3p_242930		CCGATATATCCGTCATTTTGCG
T. luce_5p_286186	ATWN01000004.1_286186	TGCCGATGGCGGCGCGACGCGT
T. luce_3p_286186		CGATGTCGCCGCCCGCCTTGGC
T. luce_5p_481971	ATWN01000009.1_481971	TAATGCATTTCGATGTCGTCTT

miRNA*	Location	Sequence
T. luce_3p_481971		CATAGAAGAACACGACAAAGGC
T. luce_5p_585272	ATWN01000012.1_585272	TGAGTTCATCAAGTGCGCGGCG
T. luce_3p_585272		CGCTTTCGCAGGCTTGATGAGC
T. luce_5p_502941	ATWN01000009.1_502941	TATGTCGATCCGGATCGTATCC
T. luce_3p_502941		TATGGTCCGCGATATTGCCCGC
T. luce_5p_123988	ATWN01000002.1_123988	AACGGTCGATCAGGACGGCAAG
T. luce_3p_123988		TTGTTCTGACCCTTTCGACCCG
T. luce_5p_252720	ATWN01000003.1_252720	TAAGGGCTGTGCGCTCCGCCGT
T. luce_3p_252720		CGCCGTCAGGATGCCGCGGGCA
T. luce_5p_313656	ATWN01000005.1_313656	ATACGATGGTTTTCCGGGCCAA
T. luce_3p_313656		ACCGGTAACCGTTCGGTTTTGG
T. luce_5p_602433	ATWN01000013.1_602433	AAACCGATGAGGTTCTTTCGAT
T. luce_3p_602433		CTTTCGATTGTTGGCGGTGCGC
T. luce_5p_589692	ATWN01000012.1_589692	TGATTTCGCCGTCGGCAAGGAT
T. luce_3p_589692		ATCCTGCCCGGTGATGGTGATC
T. luce_5p_572238	ATWN01000012.1_572238	ACACATCAAGCAAACCGGGGCG
T. luce_3p_572238		CCCGGTTTTGTTTTGTCTTCGA
T. luce_5p_559495	ATWN01000011.1_559495	GCAGGGCGACATCAACCTTGTG
T. luce_3p_559495		ACGGGTTCGCGCATTCCTATAA
T. luce_5p_27103	ATWN01000001.1_27103	GGCGCACTCACTCAAAAGGTTA
T. luce_3p_27103		CTTCTGGCAGGGCGACTCGGCG
T. luce_5p_181596	ATWN01000002.1_181596	TGTCGCGCCCGGCGTGCCCGGC
T. luce_5p_181596		ATGCCGGTCATGTTGGGCGAAA
T. luce_5p_44717	ATWN01000001.1_44717	GCCGGTAATCCGGCGGCTGTTG
T. luce_3p_44717		ATGGTTGCCTGATGACGTGCTT
T. luce_5p_184304	ATWN01000002.1_184304	CCTGTTGTTACGCGACGCATTA
T. luce_3p_184304		CGCGACGCATTAAAACCCTGTG
T. luce_5p_8020	ATWN01000001.1_8020	AAAAGCCCGGCAGCAGGACAAA
T. luce_3p_8020		CGGCCGGGCTTTTGCTGTTTTG
T. luce_5p_612357	ATWN01000014.1_612357	AATGAAAAACCCCGCAGACTGC
T. luce_3p_612357		GGGGTTTTTACGTTCTCTGGGG
T. luce_5p_599500	ATWN01000013.1_599500	ACCGCTGGGCGCGCATGGTGGT
T. luce_3p_599500		ACAGGCCTGCCTTACGTTTTAA
T. luce_5p_284324	ATWN01000004.1_284324	TAAAACAGCCCGGTGCCGTCCA
T. luce_3p_284324		CCGGGCTGTTTTGTATGTTGGG
T. luce_5p_23450	ATWN01000001.1_23450	ATGTGCCTGTTGGCGCAGATCG
T. luce_3p_23450		CAGATCGTCGAGGATTGTATTG

miRNA*	Location	Sequence
T. luce_5p_246818	ATWN01000003.1_246818	ATGCGGGTATGGGAACGCGGTG
T. luce_3p_246818		TGCCGCCGGTGTTGCTGCGGCA
T. luce_5p_186751	ATWN01000002.1_186751	TCAGGGTCAAAGATCGGATTGT
T. luce_3p_186751		CAGATTTCTTTGCCGCTCTTGG
T. luce_5p_374487	ATWN01000006.1_374487	ATCGAGGCCGCCGATACCGATC
T. luce_3p_374487		AGTTCGGCCTATATTGGCGGCT
T. luce_5p_235876	ATWN01000003.1_235876	AATATTTATGCCCGGCCTCGAT
T. luce_3p_235876		CATGATCGGTGCCGACCATGAT
T. luce_5p_46557	ATWN01000001.1_46557	CGCATGATTGATGAAGTCGGAT
T. luce_3p_46557		TTCGGCGCATTAATCATATCGT
T. luce_5p_306227	ATWN01000004.1_306227	TGCGGGCGGGGTTGGCCCCGTC
T. luce_3p_306227		TCGCAATTTCGGCAGTGCCCTG
T. luce_5p_234594	ATWN01000003.1_234594	TCATCGGACGGCCTGACTAAAC
T. luce_3p_234594		TAAACCAATAAGATGGACGATT
T. luce_5p_522493	ATWN01000010.1_522493	CGTGCCTTATACTGCACGCTCC
T. luce_3p_522493		ACGCTCCCTGCTTCGACACTTA
T. luce_5p_249012	ATWN01000003.1_249012	TGTGTGAACGAAACCGGCCTGC
T. luce_3p_249012		TGCAGGCCGGTTTGCGTTTGCA
T. luce_5p_219290	ATWN01000003.1_219290	ACAGTTGCGCTGTTTTTATGGG
T. luce_3p_219290		TTTTATGGGGGCGCTGGCGTCA
T. luce_5p_132929	ATWN01000002.1_132929	ATGTTGGCTTTGCCGTTGCTGG
T. luce_3p_132929		TTGCCGTTGCTGGTCCCCGAAG
T. luce_5p_37686	ATWN01000001.1_37686	CGTATATGTCACACGGCATGTG
T. luce_3p_37686		CGTGTGCGGCAAAGGTGATTGC
T. luce_5p_207159	ATWN01000003.1_207159	CTGGAGTTCGTGCATCGTGGGC
T. luce_3p_207159		TGCGAAGCCGAAGTCCCGCCTC
T. luce_5p_258198	ATWN01000004.1_258198	TGAGGTGTATTTCAACCCGATG
T. luce_3p_258198		TGACCATGTTGAACTCTTCCAT
T. luce_5p_345542	ATWN01000005.1_345542	CGCGGTCAGCCAAACCGGTTGA
T. luce_3p_345542		TTGTTCTGACTGCACCGCTGGT
T. luce_5p_549646	ATWN01000011.1_549646	ACAGGTCATCCACGCCTGTTTC
T. luce_3p_549646		TTTCGCTCGGTCTATGTCTGGT
T. luce_5p_394093	ATWN01000006.1_394093	TGACACTTGGCGATGGCAAAGG
T. luce_3p_394093		TCGCCTTTGCTAAAATTTCATC
T. luce_5p_354019	ATWN01000005.1_354019	ATCTTCAGGCCGGTGAGATTGT
T. luce_3p_354019		GCCGGTTTGCTGGGGTCGGGGC
T. luce_5p_593263	ATWN01000013.1_593263	AGGCTGCTGCTTCGCTCCGGGC

miRNA*	Location	Sequence
T. luce_3p_593263		CCGCGTAAGCTCAACCTCGTCG
T. luce_5p_447654	ATWN01000008.1_447654	ATAAAATCTTCCTTCTGTTTGC
T. luce_3p_447654		TGCCGGTCAGGCCGTTAAAATC
T. luce_5p_498538	ATWN01000009.1_498538	TCTGGCATCGGCGTTTCTATCG
T. luce_3p_498538		CGTCGTCTGATCCGCTTTGCCA
T. luce_5p_275552	ATWN01000004.1_275552	TCGGGCAGGGGTTGATCGGCGG
T. luce_3p_275552		TCGATACCGGGATATACGCCTG
***T. australica* NP 3b2^{T}**		
T. aust_5p_9244	JRJE01000004.1_scaffold_6_9244	CAATTAAAAACCCCGCCAGGCT
T. aust_3p_9244		CGGGGTTTTTAATTGGTAGCCG
T. aust_5p_18680	JRJE01000006.1_scaffold_4_18680	CGCCATTTTCAGGCCGAAGGCA
T. aust_3p_18680		CCGAAGGCAATGTTGTTATAGA
T. aust_5p_16396	JRJE01000005.1_scaffold_5_16396	AGGAAAACCTCAAAGCGGTTTG
T. aust_3p_16396		AAAGCGGTTTGTCTTGGCTGGG
T. aust_5p_48003	JRJE01000031.1_scaffold_1_48003	AACAGACCATCCAAGAGTCAGG
T. aust_3p_48003		AGGTGAATGGAATTGCTGTTGA
T. aust_5p_52437	JRJE01000031.1_scaffold_1_52437	CTCGTTGGTGCACCAAACGCGG
T. aust_3p_52437		AAACGCGGGCAAGTCAACGTTG
T. aust_5p_49513	JRJE01000031.1_scaffold_1_49513	CTCGATATTGTTTTGTCATTGA
T. aust_3p_49513		CAATTTAAACATTGTCACCATG
T. aust_5p_4594	JRJE01000003.1_scaffold_7_4594	TTGCCTGCAGCCCTGCCGGCCT
T. aust_3p_4594		CCTTTGCATCGGGCTTTCGTGT
T. aust_5p_11614	JRJE01000004.1_scaffold_6_11614	CCAGAGACCGAAGTCTCTGACA
T. aust_3p_11614		GACAGTTCCAGCGTTATTAGCC
T. aust_5p_6624	JRJE01000003.1_scaffold_7_6624	CGGAATGAACAACGCCGGTGGG
T. aust_3p_6624		ACCGGCGTTGTTGTTTCAAGGC
T. aust_5p_59292	JRJE01000032.1_scaffold_0_59292	TAATCTGCCCAAAGCTTGTCTT
T. aust_3p_59292		GTCTTGGCTATCATGAAGTTCA
T. aust_5p_39387	JRJE01000028.1_scaffold_12_39387	GTCATTTGGCCTGTGCCGGGCG
T. aust_3p_39387		TGCCGGGCGTTCCGCACTTGCA
T. aust_5p_42495	JRJE01000030.1_scaffold_10_42495	TTCATGGTCACCATGCTCGTCA
T. aust_3p_42495		CATGATCATCGCCAGCATGGTC
T. aust_5p_4053	JRJE01000002.1_scaffold_8_4053	AATTGCGGTATTGTTGCCATCC
T. aust_3p_4053		TTGCCATCCCGATTGCAATTGA
T. aust_5p_22590	JRJE01000008.1_scaffold_30_22590	ATGGTTGCCAAGATCATCGAAA
T. aust_3p_22590		ACGCGATCCTGCCGACCATGGG
T. aust_5p_63319	JRJE01000032.1_scaffold_0_63319	CGATGCTTACGCCGGACCCATT

miRNA*	Location	Sequence
T. aust_3p_63319		AAGCCTCATGATTAATGTGTTC
T. aust_5p_36180	JRJE01000023.1_scaffold_17_36180	CTGATTAGGAGTTGGCCGGGTT
T. aust_3p_36180		AACCCAGTTTAATCTTAGTCAT
T. aust_5p_17558	JRJE01000005.1_scaffold_5_17558	CAGATGAATGGTAAAAGGCCGT
T. aust_3p_17558		TGCGGCCTTTTATGTTTCAGGA
T. aust_5p_15365	JRJE01000005.1_scaffold_5_15365	AACGGGCGGTCGTAATGACCGC
T. aust_3p_15365		CGCCCGTTTTTCGTTTCACCAG
T. aust_5p_5875	JRJE01000003.1_scaffold_7_5875	CAAAACGAAAACAGCGCCGGAC
T. aust_3p_5875		CGGCGCTGTTTTCGTTTGTCGG
T. aust_5p_54777	JRJE01000032.1_scaffold_0_54777	CCCCGACAGAACATTCTGTCGG
T. aust_3p_54777		CGGGGCTGTTTTTTTATCTGTG
T. aust_5p_5027	JRJE01000003.1_scaffold_7_5027	CGATTATTCGCTGGCGCTTTTT
T. aust_3p_5027		CGCTTTTTTGTTTTCCGCCTCT
T. aust_5p_63384	JRJE01000032.1_scaffold_0_63384	TGAGCCTGGCGATTGGACGTCG
T. aust_3p_63384		CCGCCTGGCATCCCAGCTGACC
T. aust_5p_3291	JRJE01000002.1_scaffold_8_3291	TGAATGGCAGACGGTATATTTG
T. aust_3p_3291		AGTTTGTAAATATAACGTCTGT
T. aust_5p_56071	JRJE01000032.1_scaffold_0_56071	AGTGCCCGGTTTTTGCCGGGCA
T. aust_3p_56071		CGGGCATTTTTGTTTGTGTTTG
T. aust_5p_2750	JRJE01000002.1_scaffold_8_2750	ATTGCGTGCAAGCATCGCGATT
T. aust_3p_2750		CCCCCATTAACGGCATGAACGA
T. aust_5p_52364	JRJE01000031.1_scaffold_1_52364	TTGCTGCATCGACCGGCATTGT
T. aust_3p_52364		TTGCTCAGCCTGCCGGCGATGC
T. aust_5p_61940	JRJE01000032.1_scaffold_0_61940	CCCGCCAGTTGCCTGGCGGGGG
T. aust_3p_61940		CGGGGGTTTTGTTTCAGATCCG
T. aust_5p_56497	JRJE01000032.1_scaffold_0_56497	CACGGGTTTAATTGGCGAAGCT
T. aust_3p_56497		CTTCGCCACTTTTCATTGGGAT
T. aust_5p_39756	JRJE01000028.1_scaffold_12_39756	AGGTTAATCTGGACTTCAGCTC
T. aust_3p_39756		ACTTCAGCTCCGAAGCCGACAT
T. aust_5p_58885	JRJE01000032.1_scaffold_0_58885	AAAAACCCCGCAAGGTCACCTT
T. aust_3p_58885		GGGGTTTTTCTTTTAATTGATG
T. aust_5p_56957	JRJE01000032.1_scaffold_0_56957	TGATTCCATCCTGGCGGCCGTA
T. aust_3p_56957		AGACCCAGCTTGGTGGCGGCAT
T. aust_5p_54156	JRJE01000031.1_scaffold_1_54156	TGCCTTTTGTAATGGCAATGCC
T. aust_3p_54156		AATGCCCGAAGACGGTGCCATG
T. aust_5p_3299	JRJE01000002.1_scaffold_8_3299	ACACACACCTGATTTGGGTTTT
T. aust_3p_3299		TTTGGCCGATGATCGTGCCGGG

miRNA*	Location	Sequence
T. aust_5p_23133	JRJE01000008.1_scaffold_30_23133	GTGGAGCTGTCATTCACGGATG
T. aust_3p_23133		ACTGTAAGATGAAGATCATCCG
T. aust_5p_20162	JRJE01000006.1_scaffold_4_20162	AGCGGCTTGCGCGACGTGTTGG
T. aust_3p_20162		TGGCATGACTTCTAGCCCTGGT
T. aust_5p_37611	JRJE01000025.1_scaffold_15_37611	TCACGGCCGGTCTGTTCCTTGA
T. aust_3p_37611		CGTCATTGAACAGATCGGTGCG
T. aust_5p_57006	JRJE01000032.1_scaffold_0_57006	GTTTTGCTTGTCGGGCAACGTG
T. aust_3p_57006		AGCGTTGCCCATCAGTCAGGAT
T. aust_5p_17068	JRJE01000005.1_scaffold_5_17068	AATATCAACAACCTTGCTGCCG
T. aust_3p_17068		TTCGGTCTGCTCGGTCTTGAAG
T. aust_5p_17456	JRJE01000005.1_scaffold_5_17456	CTGGGCTTCTGGCTCGTTGGTT
T. aust_3p_17456		TGGTTTCCAGGAAGGCGTCATA
T. aust_5p_64344	JRJE01000032.1_scaffold_0_64344	TATGTCGCTGTCCTGCTGCATG
T. aust_3p_64344		CATGATATCGCCAAGGGACGCG
T. aust_5p_10528	JRJE01000004.1_scaffold_6_10528	AGCGATGGCGGCTTGTCGTCGC
T. aust_3p_10528		CGCTGGCATGGCGGATGGCGCA
T. aust_5p_968	JRJE01000001.1_968	GGTCCGGATCATTGGGCTGTCG
T. aust_3p_968		ATGCAGATTTCAACCCGAACCA
T. aust_5p_57940	JRJE01000032.1_scaffold_0_57940	AGGGAAGCTGGTTGAACAAGAC
T. aust_3p_57940		TGAGCCAGCTAGTGTCCTTTTG
T. aust_5p_7801	JRJE01000003.1_scaffold_7_7801	TCGCGCGGGCCATGACTTCCGA
T. aust_3p_7801		GACTTCCGATCAAAAAGTGCGT
T. aust_5p_9946	JRJE01000004.1_scaffold_6_9946	AAATACTCGTTTGTTCTTGCGG
T. aust_3p_9946		CAATGGGTTCAACAGCAAACAT
T. aust_5p_30915	JRJE01000020.1_scaffold_2_30915	TTCTGATCGAAGCCACGGGCGC
T. aust_3p_30915		CATCCGTGGCGTTTAACGGTTT
T. aust_5p_28718	JRJE01000009.1_scaffold_3_28718	TGGCATCGGCGGTCGCGGCATC
T. aust_3p_28718		TGGCCGGTGCGCTTTTTGCTCA
T. aust_5p_25487	JRJE01000009.1_scaffold_3_25487	AGCTCAGCCTGGTAGAGCACTG
T. aust_3p_25487		ACTGTCTTCGGGAGGCAGGGGT
T. aust_5p_35000	JRJE01000020.1_scaffold_2_35000	AACCCGACGCTGGAGATTTAGA
T. aust_3p_35000		TTTTAAAATCTCAGCTGCGGGC
T. aust_5p_16166	JRJE01000005.1_scaffold_5_16166	ATGTATGCCCGTCAGGATTGGG
T. aust_3p_16166		CTGGACGGGGTGTGCATGGAAG
T. aust_5p_37584	JRJE01000025.1_scaffold_15_37584	TCAATGCGGTGGCACCGACGGT
T. aust_3p_37584		AACTCGCTGCCGCCGCATGGTC
T. aust_5p_45945	JRJE01000031.1_scaffold_1_45945	TTCTATTTCCTGCTTATTCGTC

miRNA*	Location	Sequence
T. aust_3p_45945		AAAGCAGAAAGAGCATAAAGCA
T. aust_5p_10418	JRJE01000004.1_scaffold_6_10418	TTTGAGCAAAAGCCCATAAAGA
T. aust_3p_10418		TAAAGAATCACGTTTTGTTACC
T. aust_5p_54394	JRJE01000031.1_scaffold_1_54394	TGAAGCTTTGGACGGGGATGTT
T. aust_3p_54394		CGTTTGAATTTCTTGACGCGCG
T. aust_5p_47340	JRJE01000031.1_scaffold_1_47340	TGATGTTGTTGCCAAAAAGCTT
T. aust_3p_47340		CGTCGCATCGGTGTCTTCGATG
T. aust_5p_53512	JRJE01000031.1_scaffold_1_53512	CAAGTTCCGCTATGGCGAGACT
T. aust_3p_53512		GATCAGGTCGGTGTTGTTACCG
T. aust_5p_53204	JRJE01000031.1_scaffold_1_53204	TGATGGCGATACCATCAAGGGG
T. aust_3p_53294		ATCAAGGGGCAATGGCGCTGTT
T. aust_5p_46507	JRJE01000031.1_scaffold_1_46507	CGAAATGACAAAAGCGCACCGA
T. aust_3p_46507		GTGCGCTTTTTTCTTTTCCATC
T. aust_5p_9275	JRJE01000004.1_scaffold_6_9275	ATCGGCTTTGCTTTAAGCTCGT
T. aust_3p_9275		TCGTTCATTTTAAAGGCTTCCT
T. aust_5p_59139	JRJE01000032.1_scaffold_0_59139	CGGGCCTTCATTCGGGCGGTGG
T. aust_3p_59139		CACGAAGAAGTCCCCAGCCCGA
T. aust_5p_32539	JRJE01000020.1_scaffold_2_32539	AGGGCGAAGGCAGCGTTGCGGA
T. aust_3p_32539		CAAGTTGCTTTTGCCAATGGCG
T. aust_5p_34002	JRJE01000020.1_scaffold_2_34002	AAAGCGCAGGAGAGAAAGGTCA
T. aust_3p_34002		CTTCTCTCCTGCAGCTCGTGAT
T. aust_5p_11971	JRJE01000004.1_scaffold_6_11971	CAAAACCGGTACGACCAATGAT
T. aust_3p_11971		TTTGTTGGATTTTCGCCGGATT
T. aust_5p_23871	JRJE01000008.1_scaffold_30_23871	AACGTTCATCGTTTGGAACGTT
T. aust_3p_23871		TGTGATATATCCGCGCAAATCC
T. aust_5p_51829	JRJE01000031.1_scaffold_1_51829	CAAGGGCGTTACTGAACGGCTT
T. aust_3p_51829		CTTGGCCTGCAAATCAAGGCCG
T. aust_5p_4525	JRJE01000002.1_scaffold_8_4525	TTGATAATGCGGAATTCTCGGA
T. aust_3p_4525		ATCTGGTCGCTGTTTTCAATAA
T. aust_5p_28537	JRJE01000009.1_scaffold_3_28537	TGGTGTCGATAAAAAAGGGGAA
T. aust_3p_28537		TTCCCCTTTTTTGCGCGGAGAT
T. aust_5p_61509	JRJE01000032.1_scaffold_0_61509	ATATTTCCAACCCGCATGCGCG
T. aust_3p_61509		CGCCATGTCAGTTGGATCAGTT
T. aust_5p_42691	JRJE01000030.1_scaffold_10_42691	TTTTCCTACTGGGAACATGAGA
T. aust_3p_42691		ATGAGAACACAGTTGATATCTA
T. aust_5p_36995	JRJE01000024.1_scaffold_16_36995	ATCAGCCCATATCGTTTGATCG
T. aust_3p_36995		AATTGCCCGTTCGACATTGTCG

miRNA*	Location	Sequence
T. aust_5p_16541	JRJE01000005.1_scaffold_5_16541	TGACCCTTGTGGTTTTCACCGG
T. aust_3p_16541		CTGATTGCCCCGGGTTCCATTC
T. aust_5p_9684	JRJE01000004.1_scaffold_6_9684	AGCTGTTTCGCTTGAAACTTTG
T. aust_3p_9648		CTTTGGATTTGTCGGCAAACGC
T. aust_5p_33837	JRJE01000020.1_scaffold_2_33837	ATTTGTCCGTCTGCTGCGGTGG
T. aust_3p_33837		TTTTGGGCAAGGATGCGGTTGA
T. aust_5p_22981	JRJE01000008.1_scaffold_30_22981	AGTTGAAGCTGCGCCGGCCAGC
T. aust_3p_22981		TGACCGACGCGGCTTCTTACTC
T. aust_5p_23424	JRJE01000008.1_scaffold_30_23424	TTTGGCGATTACCCGGGCCTTT
T. aust_3p_23424		AGACCCTGGGTATTGCCATTCG
T. aust_5p_7549	JRJE01000003.1_scaffold_7_7549	CTTGGCATCACCGATGGCAAGA
T. aust_3p_7549		TGGCAAGATTGCCGCCATCGCG
T. aust_5p_40281	JRJE01000029.1_scaffold_11_40281	CTGCGTCTCCTGCAGTAATCCG
T. aust_3p_40281		AATCCGGATATCCGTAATGAGA
***T. tepidiphila* 1-1B^{T}**		
T. tepi_5p_37581	AMRN01000014.1_37581	TTGATCGGGGATCACCCCGATC
T. tepi_3p_37581		TCCTAGCTTTTTCTCTCATATC
T. tepi_5p_11218	AMRN01000002.1_11218	CTGTCGGAGACAGGGCATCTTG
T. tepi_3p_11218		CCTGCCTCCGAACAGTCTGGGA
T. tepi_5p_7722	AMRN01000001.1_7722	AGCGACATCATCGGCACTTGCG
T. tepi_3p_7722		TGTCGTTGATGTTGCTGCTAAT
T. tepi_5p_33812	AMRN01000010.1_33812	AAAGAAAAACCCCGCCAGATTG
T. tepi_3p_33812		GGGGTTTTTTGTTGGTTTTGCC
T. tepi_5p_22906	AMRN01000006.1_22906	CATTATTATTGTGTCAATTTAA
T. tepi_3p_22906		CAATTTAAACATTGTCACCATG
T. tepi_5p_9218	AMRN01000002.1_9218	CAAATCCCTTGACCGGGGATCA
T. tepi_3p_9218		TTCCTAGTTTTTTCTCTCATAT
T. tepi_5p_33714	AMRN01000010.1_33714	AGATTGATCTGACGCGCGTTGA
T. tepi_3p_33714		GTTGATGAAATTCGCCGGATGG
T. tepi_5p_24769	AMRN01000006.1_24769	AGCGACAACGCCGGTGGGATCA
T. tepi_3p_24769		TGCCACCGGCGTTGTTGTCTTC
T. tepi_5p_6580	AMRN01000001.1_6580	TCTACTTTGTCGCAAGGCATTG
T. tepi_3p_6580		CCTGCTGCCTTGCTTCGCGTAT
T. tepi_5p_9431	AMRN01000002.1_9431	AAGAAGCAGCGTCGGCCAGCCA
T. tepi_3p_9431		GCCGGCGCTGCCTCAACTCGTT
T. tepi_5p_9402	AMRN01000002.1_9402	CCGTGTACTCGACCGCTTCATC
T. tepi_3p_9402		GCGGTGCCATGGAGATCTGATG

miRNA*	Location	Sequence
T. tepi_5p_19158	AMRN01000004.1_19158	TGCAGGGTTCCTGCGCTGCCTT
T. tepi_3p_19158		CGCTGCCTTTTTTGTTGCGATT
T. tepi_5p_2570	AMRN01000001.1_2570	TACGCCGGACCCATTACTCATG
T. tepi_3p_2570		CTCATGAGTAATGTGTTCGGAA
T. tepi_5p_27975	AMRN01000007.1_27975	AATTTTCAGCCCTTGAAAATGT
T. tepi_3p_27975		AATGTCGTCATGCCGCAAATGA
T. tepi_5p_23682	AMRN01000006.1_23682	TAAAAGAAGACGGGCACCTTAT
T. tepi_3p_23682		GCCCGTCTTCTTTTGTTTTGTG
T. tepi_5p_25817	AMRN01000007.1_25817	CCGAACATCACAGCAAAACCCG
T. tepi_3p_25817		CCTTGCGGGTTTTGTTTTGCCT
T. tepi_5p_33046	AMRN01000010.1_33046	AATCATCGATCCGTTGATCTTC
T. tepi_3p_33046		AACTTTGTGCAGCTGTTTGGTC
T. tepi_5p_29936	AMRN01000008.1_29936	CGCGGCGGTGGCGTTGCCGAAC
T. tepi_3p_29936		AACGTGATGGCGTCATGCACCG
T. tepi_5p_2196	AMRN01000001.1_2196	CATGACACCGACCCGGCATTGC
T. tepi_3p_2196		TCATGCTGGGTCCAGTCGGCCT
T. tepi_5p_19433	AMRN01000004.1_19433	CGGTTGCAATTGCGACCACCAC
T. tepi_3p_19433		CCTTATAGCGGAATGCGCCCTG
T. tepi_5p_6592	AMRN01000001.1_6592	ATCAAAAAGGCGGAGCTGATTT
T. tepi_3p_6592		CTCCGCCTTTTTTTTGTTCGAG
T. tepi_5p_7069	AMRN01000001.1_7069	AAGGGCGGTAAACCGTTCTTTG
T. tepi_3p_7069		CTTTGGCATCTGTGTTGGCATG
T. tepi_5p_3446	AMRN01000001.1_3446	TCAGTGCCGCGCACTTCGTCGT
T. tepi_3p_3446		AGTGCGGCATCGACCTGATCAC
T. tepi_5p_36487	AMRN01000013.1_36487	GGGGGGAAAAGTTCCCTTGCCG
T. tepi_3p_36487		TTGCCGAACGGCTGAAAGAGCT
T. tepi_5p_28590	AMRN01000008.1_28590	CATTCTGGCGCTATGCCGGTAT
T. tepi_3p_28590		TATGCGTTTTGTCCATGACAAA
T. tepi_5p_19555	AMRN01000004.1_19555	CAATCTGTTGCAGTGCCTGATC
T. tepi_3p_19555		CTGATCTGCTTCGTTACGGATA
T. tepi_5p_5471	AMRN01000001.1_5471	TGCCTATCGCGTCGACGAGGTG
T. tepi_3p_5471		TGTCGAGGCGGCTGGTCTGCGT
T. tepi_5p_572	AMRN01000001.1_572	CACCGATGTCGAAAGATCTTCG
T. tepi_3p_572		TTCCATATCGTGTCGTTATTCA
T. tepi_5p_20938	AMRN01000005.1_20938	AATTGTATGTGCAATAATGCGA
T. tepi_3p_20938		GCGTTCGGAGGATTGCACATGC
T. tepi_5p_22942	AMRN01000006.1_22942	CTGCGACACAGTTGTCGCAGGC

miRNA*	Location	Sequence
T. tepi_3p_22942		TGGGGTGGCTTTTCTTTTTCGG
T. tepi_5p_34995	AMRN01000011.1_34995	CAAATCCCTTGACCTGGGATCA
T. tepi_3p_34995		ATTTTCCTAGCTTTTTCTCTCA
T. tepi_5p_5603	AMRN01000001.1_5603	CGCCTATTGGAATTCGGACGCG
T. tepi_3p_5603		CGGACGCGATGCTGCTGCGTAT
T. tepi_5p_30879	AMRN01000008.1_30879	TTTCGACAATGACGATGTGTCG
T. tepi_3p_30879		TGGCGTCGTTGCCGAAAGCTGG
T. tepi_5p_11782	AMRN01000002.1_11782	TGTGATGGTTTCTTCTATCGCA
T. tepi_3p_11782		GTCGGTGGCGGTAACACCGCGG
T. tepi_5p_25251	AMRN01000007.1_25251	TTTCAACAACGCCCGTTGATTG
T. tepi_3p_25251		ATTGAAATCCCCCGCCTAAACC
T. tepi_5p_27927	AMRN01000007.1_27927	ATTTTGTACCTGATGAAACGGC
T. tepi_3p_27927		CGTTTTGTTAGGTGTTAACCTG
T. tepi_5p_17905	AMRN01000004.1_17905	AAGATGTCCGTAATTCGCGCGG
T. tepi_3p_17905		CATCAACCTGTTGAACGGGTTG
T. tepi_5p_21204	AMRN01000005.1_21204	ATCGTCACCTTTGATGGTAACC
T. tepi_3p_21204		TAACCTTTACGAAGCCATTGCC
T. tepi_5p_15757	AMRN01000003.1_15757	TATGCCAACAATCCGACCGGGT
T. tepi_3p_15757		GCGGTCTGGATGTTGGCCTGCC
T. tepi_5p_26936	AMRN01000007.1_26936	AACAAAACCCGCAAGGCCAATG
T. tepi_3p_26936		TTGCGGGTTTTGCTGTGATGTT
T. tepi_5p_29436	AMRN01000008.1_29436	AAAGGTGTATTTGTTGAAGACG
T. tepi_3p_29436		TGCGTTCTTCAGCGCACCTTCA
T. tepi_5p_16459	AMRN01000003.1_16459	AACGGCGTTGGTTGAAACGGTT
T. tepi_3p_16459		TTTCAGGATGAAAATGACCGCC
T. tepi_5p_18168	AMRN01000004.1_18168	AAAGGCATCTATATGCCGGTCG
T. tepi_3p_18168		GGTCGGTCTCCTTGGCTTTCCT
T. tepi_5p_17794	AMRN01000004.1_17794	CTGAGCCTTGAGGCCTTTGGCG
T. tepi_3p_17794		TTGGCGGTGATCTTCATGACTT
T. tepi_5p_13698	AMRN01000003.1_13698	AGCAAAAGCTGCCTAATTAAGG
T. tepi_3p_13698		CTTCTGCTTTACAGACAGAATT
T. tepi_5p_21300	AMRN01000005.1_21300	TGCGCGACGATCATCATCACGG
T. tepi_3p_21300		GATCATGATGATCATCGTCGTG
T. tepi_5p_31639	AMRN01000009.1_31639	CAGCGGCCTTGTTGATCCGCTT
T. tepi_3p_31639		CGCTTATCTATTTGCCGTGATC
T. tepi_5p_17561	AMRN01000004.1_17561	AACTGGTCTCGGTTCTGACCTG
T. tepi_3p_17561		ATGTCGAAACCGAAACCATGCC

miRNA*	Location	Sequence
T. tepi_5p_33271	AMRN01000010.1_33271	TGTCTTTTTCTGACGTTTTTTC
T. tepi_3p_33271		CGTTTTTTCTCAAAAAAGGGTT
T. tepi_5p_11264	AMRN01000002.1_11264	TTGTCTGTCAAACAGGCAAGGA
T. tepi_3p_11264		AAGGATTGCGGTCGGCCTTACT
T. tepi_5p_8741	AMRN01000002.1_8741	TGCAGTTCGATGAAGTTCTTCT
T. tepi_3p_8741		CCGACCTTTTCCAAGGATGACA
T. tepi_5p_34376	AMRN01000011.1_34376	TGACGCAGAGGCTTTCTCTCAT
T. tepi_3p_34376		AGGTGGCCTTTGGATCACCCGG
T. tepi_5p_33867	AMRN01000010.1_33867	GTCCGACTGGCTTGGATAAATC
T. tepi_3p_33867		AGCTGTAATCACTGCGCACCAT
T. tepi_5p_29208	AMRN01000008.1_29208	CCGGTGCTTCTGTGTGCCGACG
T. tepi_3p_29208		ATGGCTGACATTCCGCGTCTGG
T. tepi_5p_19094	AMRN01000004.1_19094	TTCAAGGATTATCTTGCCACAC
T. tepi_3p_19094		TTTGGCGAGATGGGCCTTGCCG
T. tepi_5p_8405	AMRN01000002.1_8405	GTCGGCGTTGTCGCGCTGTTCA
T. tepi_3p_8405		TTCAAGGAGCCGCTGCATGTTG
T. tepi_5p_8120	AMRN01000001.1_8120	TGCGCCGTTCGGCCTATGTCGC
T. tepi_3p_8120		CACCGTTTTGATGCCGTCCTTT
T. tepi_5p_33254	AMRN01000010.1_33254	TGCTGGAACAGCATTTCGGTGT
T. tepi_3p_33254		TTCGGTGTTTGCGACTTCAAAG
T. tepi_5p_18865	AMRN01000004.1_18865	TGTCGGGCGGGCGCGGCGGTGT
T. tepi_3p_18865		CAATGAGCCCTATATCGCGGTG
T. tepi_5p_2711	AMRN01000001.1_2711	CCGGATATAACTATCCGTCGGG
T. tepi_3p_2711		TCGGGCAGCACTTGTTGGCCGG
T. tepi_5p_5680	AMRN01000001.1_5680	AGGGTGCAGAAGCATTCGTTCG
T. tepi_3p_5680		GTTCGCCTTTTTTATAAGGATG
***T. xiamenensis* M-5^{T}**		
T. xiam_5p_26826	CP004388.1_26826	AGACGTGACCTTCGGGTCGCGT
T. xiam_3p_26826		CGTCTTTTTTATTGTCTGGTGG
T. xiam_5p_11702	CP004388.1_11702	CAATTAAAAACCCCCTCAGGCG
T. xiam_3p_11702		AGGGGTTTTTTAATTGGTAGCC
T. xiam_5p_37696	CP004388.1_37696	TCATTTTTGCGCCGACCCTATT
T. xiam_3p_37696		CGACCCTATTCATTTCTGAATT
T. xiam_5p_39114	CP004389.1_39114	GATATCAGACCTTACCTGCACC
T. xiam_3p_39114		CGGTGCAGGTAAGGTCTGTAGG
T. xiam_5p_27030	CP004388.1_27030	CGCGGTGAGATTTTCGCCGCGT
T. xiam_3p_27030		CGCGTTTTTTGTCGGTACCGCA

miRNA*	Location	Sequence
T. xiam_5p_6808	CP004388.1_6808	GCGCGGGAATGACGGTTTTTGG
T. xiam_3p_6808		TCACTCCCGCGAAGGCGGGAGT
T. xiam_5p_32390	CP004388.1_32390	CGGTTGCATTGGCAAAGGCATC
T. xiam_3p_32390		AGGCATCGACGGATGCCGGTGC
T. xiam_5p_34788	CP004388.1_34788	TTATACATTGAGAGGCAGAAGG
T. xiam_3p_34788		CTTCTGCCTCTTTTTCGTTTGA
T. xiam_5p_8112	CP004388.1_8112	TGCCAGCCATCGAAAACCGATT
T. xiam_3p_8112		GCCGGTTTTCTGTGGCTGATTT
T. xiam_5p_12450	CP004388.1_12450	ATCCGTCAGCGCGTTCTGGCTG
T. xiam_3p_12450		CTGGATTATTCGCAACGCTGCT
T. xiam_5p_17371	CP004388.1_17371	CTTTTCCATCTGTTTGGCGGCA
T. xiam_3p_17371		TTATTCAGATCTGGAAAAGCTG
T. xiam_5p_6543	CP004388.1_6543	TATAAAAGGCCCGCCGTTCTGG
T. xiam_3p_6543		AGAATTGCGGGTCTTTTTTGGT
T. xiam_5p_18736	CP004388.1_18736	ATCGCCCGCCATTCGGGTGCTT
T. xiam_3p_18736		TCCGAAATGCGTTTGTCGCGAA
T. xiam_5p_38869	CP004388.1_38869	AGATTCGTTTTTTATCTATAAT
T. xiam_3p_38869		TATCTATAATTTTGAATGCGAT
T. xiam_5p_35854	CP004388.1_35854	AGTGCGGATCGCGCCTCGATGC
T. xiam_3p_35854		CGGGGCCGGATCCGTTGACGAG
T. xiam_5p_36112	CP004388.1_36112	ACGAAACGAAAGACCCCCATTA
T. xiam_3p_36112		TGGGGGTCTTTGCGTTTCAGGT

*: The identified putative miRNAs were named according to <name of bacteria>_<location of miRNA in the precursor>_<position of miRNA in the genome>. All sequences have 22 nt in length.

Appendix 2 Alignment of bacterial putative miRNAs of the genus *Thalassospira* with ≥ 50% similarity to those of salt responsive barley miRNAs.

Full sequence-related miRNAs		Sequence alignment				% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**					
T. luce_5p_21030	hvu-MIR171	hvu-MIR171	1	TGTTGGCT-CGACTCACTCAGA	21	50.0
				|||..|.. |.||.|..|..||		
		T.luce_5p_21030	1	TGTGCGAGGCAACACGTTGCGA	22	
T.luce_3p_252989		hvu-MIR171	1	TGTTG-GCTCGACTCACTCAGA	21	59.0
				.|.|| |.||||.|...|||.		
		T.luce_3p_252989	1	CGATGCGTTCGACACCGCCAGC	22	
T. aust_3p_39387		hvu-MIR171	1	TGTTGG-CTCGACTCACTCAGA	21	50.0
				||..|| |....|.|||...|		
		T.aust_3p_39387	1	TGCCGGGCGTTCCGCACTTGCA	22	
T. luce_5p_181596		hvu-MIR171	1	TGTTG-GCTCGACTCACTCAGA	21	54.5
				|||.| ||.||.|...|.|.|.		
		T.luce_5p_181596	1	TGTCGCGCCCGGCGTGCCCGGC	22	
T.tepi_5p_9402		hvu-MIR171	1	TGTTGG-CTCGACTCACTCAGA	21	50.0
				...||. |||||...|||..		
		T.tepi_5p_9402	1	CCGTGTACTCGACCGCTTCATC	22	
T.profu_3p_56451	hvu-MIR159a/b	hvu-MIR159a/b	1	TTTGGATTG-AAGGGAGCTCTG	21	50.0
				|.|...||. ||.|||..||..		
		T.profu_3p_56451	1	TATCTTTTTCAAAGGAAGTCAT	22	
T.profu_3p_38494		hvu-MIR159a/b	1	---TTTGGATTGAAGGGAGCTCTG	21	50.0
				|.||| |.||||..|.||..		
		T.profu_3p_38494	1	ACATGTGG--TCAAGGTTGTTCGA	22	

Full sequence-related miRNAs		Sequence alignment				% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**					
T.luce_3p_432525		hvu-MIR159a/b	1	TTTGGATTGAAGGGAGCT-CTG	21	54.5
				|..||..|.||..|.|.| |||		
		T.luce_3p_432525	1	TGCGGTGTCAAACGGGGTGCTG	22	
T.xian_3p_3489		hvu-MIR159a/b	1	TTTGGATTGAA-GGGAGCTCTG	21	54.5
				......|.||. |||||||.|		
		T.xian_3p_3489	1	AAACCGTCGACCGGGAGCTCGG	22	
T.luce_3p_602433		hvu-MIR159a/b	1	-TTTGGATTGAAGGGAGCTCTG	21	54.5
				|||.|||||..||..|..|..		
		T.luce_3p_602433	1	CTTTCGATTGTTGGCGGTGCGC	22	
T.aust_3p_9648		hvu-MIR159a/b	1	-TTTGGATTGAAGGGAGCTCTG	21	50.0
				||||||||....||....|..		
		T.aust_3p_9648	1	CTTTGGATTTGTCGGCAAACGC	22	
T.aust_5p_57006		hvu-MIR159a/b	1	-TTTGGATTGAAGGGAGCTCTG	21	54.5
				|||.|.|||..|||.....||		
		T.aust_5p_57006	1	GTTTTGCTTGTCGGGCAACGTG	22	
T.tepi_3p_19433/ T.profu_3p_29989		hvu-MIR159a/b	1	T-TTGGATTGAAGGGAGCTCTG	21	50.0
				. ||..|..|.|..|.||.|||		
		T.tepi_3p_19433	1	CCTTATAGCGGAATGCGCCCTG	22	
T.profu_3p_33610	hvu-MIR5048a/b	hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	54.5
				.||||.|.|.|.||...|||..		
		T.profu_3p_33610	1	CATTTCCGGATCTTTTTTCTGT	22	
T.profu_5p_56952		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	54.5
				.||||.|...|.|...|||.||		
		T.profu_5p_56952	1	AATTTACCCCTGTCCCGTCCAA	22	

Full sequence-related miRNAs		Sequence alignment				% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**					
T.xian_3p_22881		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	50.0
				|||||...||.||..|.....|		
		T.xian_3p_22881	1	TATTTATCGGGTTGGGCGGCGA	22	
T.xian_5p_16719		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	50.0
				..||||.|.|..||..||.|..		
		T.xian_5p_16719	1	ATTTTGGATGCCTTGCGTGTTT	22	
T.aust_3p_3291		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	54.5
				..||||.|..|.|.|.||||..		
		T.aust_3p_3291	1	AGTTTGTAAATATAACGTCTGT	22	
T.luce_3p_249012		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	50.0
				|....||.|||||..|.|...|		
		T.luce_3p_249012	1	TGCAGGCCGGTTTGCGTTTGCA	22	
T.xiam_3p_34788		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	54.5
				..|.|||...||||..||.|.|		
		T.xiam_3p_34788	1	CTTCTGCCTCTTTTTCGTTTGA	22	
T.xiam_5p_17371		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	50.0
				..|||.||..|.||.||....|		
		T.xiam_5p_17371	1	CTTTTCCATCTGTTTGGCGGCA	22	
T.tepi_3p_34995		hvu-MIR5048a/b	1	TATTTGCAGGTTTTAGGTCTAA	22	54.5
				..|||.|..|.|||...|||.|		
		T.tepi_3p_34995	1	ATTTTCCTAGCTTTTTCTCTCA	22	

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.xiam_3p_38869		hvu-MIR5048a/b 1 TATTTGCAGGTTTTAGGTCTAA 22 |||.|..|..|||.|...|.|. T.xiam_3p_38869 1 TATCTATAATTTTGAATGCGAT 22	50.0
T.aust_3p_33837		hvu-MIR5048a/b 1 TATTTG-CAGGTTTTAGGTCTAA 22 |.||.| ||.|..|..||| |.| T.aust_3p_33837 1 TTTTGGGCAAGGATGCGGT-TGA 22	56.5
T.alka_5p_329	SUT_hvu_mir_000173	SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 .||.||||.||.|.|....|. T.alka_5p_329 1 GGATTAGGAGGCTGGCAACAAC 22	54.5
T.profu_5p_7879		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 .|.|||...||.||.||..|.. T.profu_5p_7879 1 TGCCTATCGCGTCGACGAGGTG 22	50.0
T.profu_5p_3800		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 |..|.|.|....|||||... T.profu_5p_3800 1 ACCCGATGCTTACGCCGGACCC 22	50.0
T.xian_5p_25207		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGA-A 22 |.|..|||.|....||.|.| | T.xian_5p_25207 1 AAAGCAGGAAGAATACGAACAGA 23	52.2
T.luce_3p_523350		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 .|||.|.|||...|.|...||. T.luce_3p_523350 1 TGACCATGACCATGACCATGAC 22	50.0
T.luce_5p_544598		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 |.|..|...|||||.|.... T.luce_5p_544598 1 AAAAGAAACCGCCGCCCGGCTT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_589692		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 .||.|..|.||.||.|...||. T.luce_5p_589692 1 TGATTTCGCCGTCGGCAAGGAT 22	50.0
T. luce_5p_617474		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 .||.||..|||.|..|...|.| T.luce_5p_617474 1 TGATTACAACGGCTGCACGGCA 22	50.0
T.tepi_5p_5471		SUT_hvu_mir_000173 1 AGACTAGGACGCCGCCGGAGAA 22 .|.|||...||.||.||..|.. T.tepi_5p_5471 1 TGCCTATCGCGTCGACGAGGTG 22	50.0
T.profu_5p_15457	SUT_hvu_mir_000108	SUT_hvu_mir_000108 1 CACGAGGGCTCTGCTCGCTGAT 22 |.|.|||.....|.|||.||. T.profu_5p_15457 1 CGCAAGGAAAAGCCCCGCAGAC 22	50.0
T.xian_3p_19488		SUT_hvu_mir_000108 1 CACGAGGGCTCTGCTCGCTGAT 22 ||||.|..||.....||.||.. T.xian_3p_19488 1 CACGCGAACTGATGGCGATGCC 22	50.0
T.luce_3p_198770		SUT_hvu_mir_000108 1 CACGAGGGCTCTGCTCGCTGAT 22 ||.|..|.|..|.|.||.||.| T.luce_3p_198770 1 CATGTTGCCGTTTCGCGATGGT 22	54.5
T.luce_5p_22792		SUT_hvu_mir_000108 1 CACGAGGGCTCTGCTCGCTGAT 22 |....|.|.||||.||.... T.luce_5p_22792 1 CGATTGTGTTCTGCTTGCGTCG 22	50.0
T.luce_3p_235876		SUT_hvu_mir_000108 1 CACGAGGGCTCTGCTCGCTGAT 22 ||.||..|.|.....|..||| T.luce_3p_235876 1 CATGATCGGTGCCGACCATGAT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.aust_3p_16396		SUT_hvu_mir_000108 1 CACGAGGGCTCTGCTCGCTGAT 22 .|.|.||..|.|..|.||||.. T.aust_3p_16396 1 AAAGCGGTTTGTCTTGGCTGGG 22	50.0
T.xiam_3p_12450		SUT_hvu_mir_000108 1 CACGAGGGCTCTGCTCGCTGAT 22 |..||....||....||||.| T.xiam_3p_12450 1 CTGGATTATTCGCAACGCTGCT 22	50.0
T.aust_5p_7801		SUT_hvu_mir_000108 1 -CACGAGGGCTCTGCTCGCTGAT 22 |.||.||||..||....|.|| T.aust_5p_7801 1 TCGCGCGGGCCATGACTTCCGA- 22	52.2
T.profu_3p_44551	SUT_hvu_mir_000174	SUT_hvu_mir_000174 1 TT-GCATCTCTCGGGTCGTTCCAG 23 .| || ||.||||.|.|||...| T.profu_3p_44551 1 CTAGC--CTTTCGGATGGTTTGCG 22	58.3
T.luce_5p_528636		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 || |....|||..||..||..|| T.luce_5p_528636 1 TT-CGGTGCTCACGTACTTTTAG 22	52.2
T.luce_5p_528656/ T.luce_5p_528636		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 || |....|||..||..||..|| T.luce_5p_528656 1 TT-CGGTGCTCACGTACTTTTAG 22	52.2
T.luce_5p_38961		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 ..| |.|..|..|||..|.|||| T.luce_5p_38961 1 CAG-ACCATTTTGGTTCTGCCAG 22	52.2
T.luce_5p_531055		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 ||||. ||.|||..|||...|.| T.luce_5p_531055 1 TTGCG-CTATCGATTCGCAACTG 22	60.9

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_3p_544598		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 |.||.|.| ||...|...|||.| T.luce_3p_544598 1 TGGCGTTT-TCTTTTGTGTCCTG 22	52.2
T. luce_3p_306227		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 |.|||..| ||||......||.| T.luce_3p_306227 1 TCGCAATT-TCGGCAGTGCCCTG 22	52.2
T.luce_3p_132929		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 ||||...|. |.||||.....|| T.luce_3p_132929 1 TTGCCGTTG-CTGGTCCCCGAAG 22	52.2
T.tepi_5p_17561		SUT_hvu_mir_000174 1 TTGCATCTCTCGGGTCGTTCCAG 23 .. |...||||||.||...||.| T.tepi_5p_17561 1 AA-CTGGTCTCGGTTCTGACCTG 22	52.2
T.profu_3p_778/ T.tepi_3p_572	SUT_hvu_mir_000045	SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 |||....|..||..|||||||. T.profu_3p_778 1 --TTCCATATCGTGTCGTTATTCA 22	54.2
T.profu_3p_24235/ T.tepi_3p_15757		SUT_hvu_mir_000054 1 GCTTCTTGCTGATGGTGTTATTCC 24 ||....|| ||||.||...|.|| T.profu_3p_24235 1 GCGGTCTG--GATGTTGGCCTGCC 22	54.2
T.xian_3p_19488		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 ....|...|||||||.| ||.|| T.xian_3p_19488 1 CACGCGAACTGATGGCG--ATGCC 22	54.2
T.xian_3p_3333		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 .....|.||||..|||| ||.|| T.xian_3p_3333 1 AGCCATCGCTGCCGGTG--ATCCC 22	54.2

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_3p_394944		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 |.|....|.||.||.|| ||.|. T.luce_3p_394944 1 GGTGACGGTTGTTGTTG--ATCCG 22	50.0
T.aust_5p_54156		SUT_hvu_mir_000045 1 -GCTTCTTGCTGATGGTGTTATTCC 24 ||.|.||| |.||||. .||.|| T.aust_5p_54156 1 TGCCTTTTG-TAATGGC--AATGCC 22	60.0
T.aust_3p_53512		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 |.| |..|..|.||.|||||. |. T.aust_3p_53512 1 GAT-CAGGTCGGTGTTGTTAC-CG 22	54.2
T.tepi_3p_8120		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 |..|.|..|||||..||..||. T.tepi_3p_8120 1 -CACCGTTTTGATGCCGTCCTTT- 22	50.0
T.xiam_3p_34788		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 |||||..||..|..|..| ||.. T.xiam_3p_34788 1 -CTTCTGCCTCTTTTTCGT-TTGA 22	50.0
T.tepi_3p_21300		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 |.| |.||.||||..|..|..|. T.tepi_3p_21300 1 GAT-CATGATGATCATCGTCGTG- 22	50.0
T.xian_3p_16668/ T.xiam_3p_26826		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 |.||||..|.||.||.|..|.. T.xian_3p_16668 1 -CGTCTTTTTTATTGTCTGGTGG- 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T. tepi_3p_7722		SUT_hvu_mir_000045 1 GCTTCTTGCTGATGGTGTTATTCC 24 ..|..|||.||.||.||.| T.tepi_3p_7722 1 TGTCGTTGATGTTGCTGCTAAT-- 22	54.2
T.alka_3p_4942 / T.luce_3p_520914	SUT_hvu_mir_000163	SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 |||| || ||.||...|..|||.. T.alka_3p_4942 1 CATA-AT-TAATGTGTTCGGAACT 22	58.3
T.profu_3p_30109/ T.tepi_3p_19555		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 |..||.||. |.|..||.|.|.| T.profu_3p_30109 1 CTGATCTGC--TTCGTTACGGATA 22	50.0
T.profu_5p_32260/ T.tepi_5p_20938		SUT_hvu_mir_000163 1 CATATATGTA-GTGCTGTAAGAAGA 24 .|| |||| ||||..|||...|| T.profu_5p_32260 1 AAT---TGTATGTGCAATAATGCGA 22	60.0
T.luce_3p_520914		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 |||| || ||.||...|..|||.. T.luce_3p_520914 1 CATA-AT-TAATGTGTTCGGAACT 22	58.3
T.xian_3p_22881		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 .||.|||...|| ||...|..|| T.xian_3p_22881 1 TATTTATCGGGT--TGGGCGGCGA 22	50.0
T.luce_3p_314181		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 |||.|||.|...|.| |||.|.. T.luce_3p_314181 1 CATCTATCTGAAGTT--AAGTAAG 22	54.2
T.luce_3p_546117		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 .|.. |||..|..|..|||||. T.luce_3p_546117 1 AAGG--TGTTATCGTCGAAGAAGG 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.aust_5p_40281		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 | |...|.|..|||.||||...| T.aust_5p_40281 1 C-TGCGTCTCCTGCAGTAATCCG- 22	50.0
T.tepi_5p_29436		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 |.|..||||.|..|..||||.| T.tepi_5p_29436 1 -AAAGGTGTATTTGTTGAAGACG- 22	54.2
T.tepi_5p_34995		SUT_hvu_mir_000163 1 CATATATGTAGTGCTGTAAGAAGA 24 ||.||...|.|..||| .||..| T.tepi_5p_34995 1 CAAATCCCTTGACCTG--GGATCA 22	50.0
T.profu_5p_44441	SUT_hvu_mir_000150	SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT- 22 ..||.| |..||.||||.|..| T.profu_5p_44441 1 AGTGTA-CTCAGTGAGTATGCTC 22	52.2
T.luce_3p_14713		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 ||||.. |||.|||....|..| T.luce_3p_14713 1 TTTGCG-CGATGGGTCCCTGAT 21	54.5
T. xian_5p_23642		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 |.|||..|....|..|.||.|| T.xian_5p_23642 1 TCTGGGCCTGCCGACGGTTCTT 22	50.0
T. luce_3p_350700		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 ||.||..||....|..|||.|| T.luce_3p_350700 1 TTCGGCCCGGTTTGCATTTGTT 22	54.5
T.profu_5p_50983		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 |.|||.......|.|||.|||. T.profu_5p_50983 1 TATGGCGTTGCTGTAGTGTTTG 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_528656/ T.luce_5p_528636		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 ||.||..|..|.|.|.|||.. T.luce_5p_528656 1 TTCGGTGCTCACGTACTTTTAG 22	54.5
T.luce_3p_312757		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 |||.....||.||....||| T.luce_3p_312757 1 TTTTACCGTAATGGTTGGTTTT 22	50.0
T.luce_5p_186751		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 |..||.||.||...|..||.| T.luce_5p_186751 1 TCAGGGTCAAAGATCGGATTGT 22	54.5
T.luce_3p_549646		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 |||.|.|||.....||.|..| T.luce_3p_549646 1 TTTCGCTCGGTCTATGTCTGGT 22	50.0
T.tepi_5p_8741		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 |...|.|||.|....|.||.| T.tepi_5p_8741 1 TGCAGTTCGATGAAGTTCTTCT 22	50.0
T.tepi_3p_19094		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 |||||...||.|||..||.... T.tepi_3p_19094 1 TTTGGCGAGATGGGCCTTGCCG 22	54.5
T.aust_5p_30915		SUT_hvu_mir_000150 1 TTTGGATCGAAGGGAGTTTTTT 22 ||..|||||||..|....... T.aust_5p_30915 1 TTCTGATCGAAGCCACGGGCGC 22	50.0
T.profu_5p_43344	SUT_hvu_mir_000133	SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 ....|.|||.|||||..||| T.profu_5p_43344 1 ATCTGTTTTGGGGCGATCCGAA- 22	60.9

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.profu_5p_44486		SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 |.|||..||||.||||...||. T.profu_5p_44486 1 GGACGCATTGATGCGACCCGAG- 22	60.9
T.luce_3p_384973		SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 ...||.||||.|.||||....| T.luce_3p_384973 1 TGTCGTTTTGTGCCGATAACGA- 22	52.2
T.xian_3p_16719		SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 |||.||.||.|. |..||||... T.xian_3p_16719 1 GAAGGAATTCAT-CATTTTGCCA 22	56.5
T.luce_5p_167793		SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 .|.|.|.|.||....|||||.| T.luce_5p_167793 1 TATCCACTAGACATCATTTGGA- 22	52.2
T.luce_3p_314181		SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 .|.|.||.|||.|..|..| ||. T.luce_3p_314181 1 CATCTATCTGAAGTTAAGT-AAG 22	52.2
T.luce_3p_498538		SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 ...|| |.|||..||.||||... T.luce_3p_498538 1 CGTCG-TCTGATCCGCTTTGCCA 22	52.2
T.luce_3p_354019		SUT_hvu_mir_000133 1 GAACGATTTGAGGCGATTTGAAC 23 |. ||.||||..|.|.|..|..| T.luce_3p_354019 1 GC-CGGTTTGCTGGGGTCGGGGC 22	52.2

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_602433		SUT_hvu_mir_000133 1 GAAC-GATTTGAGGCGATTTGAAC 23 .||| || ||||...|||..|. T.luce_5p_602433 1 AAACCGA--TGAGGTTCTTTCGAT 22	58.3
T. profu_5p_9602	SUT_hvu_mir_000075	SUT_hvu_mir_000075 1 AAGGAAACTGGGGCAGTGGCATAT 24 |...|||..|.||...|| ||.| T.profu_5p_9602 1 ATCAAAAAGGCGGAGCTG--ATTT 22	50.0
T.alka_5p_4279		SUT_hvu_mir_000075 1 AAGGAAACTGGGGCAGTGGCATAT 24 .|.|....|..| |.|||. ||| T.alka_5p_4279 1 CAAGGTGGTAAG-CGGTGGT-TAT 22	50.0
T.luce_5p_525349		SUT_hvu_mir_000075 1 AAGGAAACTGGGG-CAGTGGCATAT 24 ||| |..|||.|| ||.| ||||| T.luce_5p_525349 1 AAG-AGCCTGTGGACACT--CATAT 22	68.0
T.luce_3p_58702		SUT_hvu_mir_000075 1 AAGGAAACTGGGGCAGTGGCATAT 24 |.|. .|||...|||.|||... T.luce_3p_58702 1 ACGT--CCTGATTCAGTTGCAGCG 22	50.0
T.luce_3p_559495		SUT_hvu_mir_000075 1 AAGGAAACTGGGGCAGTGGCATAT 24 |.||.. |.|.|||.|...|||. T.luce_3p_559495 1 ACGGGT--TCGCGCATTCCTATAA 22	50.0
T.luce_5p_399890		SUT_hvu_mir_000075 1 AAGGAAACTGGGGCAGTGGCATAT 24 | .||||.||..||.|.|.||.. T.luce_5p_399890 1 A--TAAACCGGCCCAATCGGATGC 22	54.2
T.tepi_5p_24769		SUT_hvu_mir_000075 1 AAGGA-AACTGGGGCAGTGGCATAT 24 |..|| ||| |.|.|||.||.. T.tepi_5p_24769 1 AGCGACAAC---GCCGGTGGGATCA 22	56.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.alka_3p_4279	SUT_hvu_mir_000049	SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 .|..|||.||...||..||||. T.alka_3p_4279 1 ATAACCGCCGCTTTCTCCTTTC 22	54.5
T.alka_3p_1819		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 .|..||.|.|.||.||...|.| T.alka_3p_1819 1 ATGACCATGGCCGACAAAATCG 22	50.0
T. luce_3p_399471		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 ||....||.||..|.||...|| T.luce_3p_399471 1 TTGCTGGTTGATTTTATGCATG 22	50.0
T.profu_5p_12216		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 .||..|||.|.||...|.||.. T.profu_5p_12216 1 GTCGGCGTTGTCGCGCTGTTCA 22	50.0
T.profu_5p_50983		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 |....|||.|..||..|.||| T.profu_5p_50983 1 TATGGCGTTGCTGTAGTGTTTG 22	54.5
T.luce_5p_528656/ T. luce_5p_528636		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 |||...|...|||..|.||.| T.luce_5p_528656 1 TTCGGTGCTCACGTACTTTTAG 22	54.5
T.luce_3p_559608		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 ||...|||.|.|..|..||.|. T.luce_3p_559608 1 TTTGGCGTTGCCTACGCCTATT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T. xian_5p_22099		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 ||..|.|..|...||||.|.. T.xian_5p_22099 1 TTGCCGGCTGTGATCATCATGT 22	50.0
T. luce_3p_218949		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 ..|...|.|||...|||.|.| T.luce_3p_218949 1 ACCATGGCCGACAAAATCGTCG 22	50.0
T.luce_5p_167793		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 |...|..|.|||.|||.|... T.luce_5p_167793 1 TATCCACTAGACATCATTTGGA 22	50.0
T.aust_5p_42495		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 |||...|||..|.|..||.|.. T.aust_5p_42495 1 TTCATGGTCACCATGCTCGTCA 22	50.0
T.luce_3p_422620		SUT_hvu_mir_000049 1 TTC--TCCGTCGACGTCATCTTTG 22 ..| ||| |.||.|||.||| T.luce_3p_422620 1 GACGATCCG--GGCGACATCCTTG 22	62.5
T.aust_5p_3291		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 |.....|..||||..||.|||| T.aust_5p_3291 1 TGAATGGCAGACGGTATATTTG 22	54.5
T.aust_3p_52437		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 .....||...|.||||.|.||| T.aust_3p_52437 1 AAACGCGGGCAAGTCAACGTTG 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.tepi_5p_8405		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 .||..|||.|.||...|.||.. T.tepi_5p_8405 1 GTCGGCGTTGTCGCGCTGTTCA 22	50.0
T.profu_3p_37885/ T.tepi_3p_24769		SUT_hvu_mir_000049 1 TTCT-CCGTCGACGTCATCTTTG 22 |.|. |||.||..||..||||. T.profu_3p_37885 1 TGCCACCGGCGTTGTTGTCTTC- 22	56.6
T.tepi_3p_33254		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 |||...||...||.|.||...| T.tepi_3p_33254 1 TTCGGTGTTTGCGACTTCAAAG 22	50.0
T.aust_5p_9684		SUT_hvu_mir_000049 1 TTCTCCGTCGACGTCATCTTTG 22 ..||...|||.....|.|||| T.aust_5p_9684 1 AGCTGTTTCGCTTGAAACTTTG 22	50.0
T.profu_5p_33977	SUT_hvu_mir_000050	SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 .|.||||..|..|||..|||| T.profu_5p_33977 1 CATCAGAGATGCAGGGCCTATT 22	63.6
T.profu_3p_54600		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 |..|.|...|||.||.|..||. T.profu_3p_54600 1 ACGCGGCGGTCATGGCTGGATG 22	50.0
T.xian_3p_3489		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 ||||.|....|..|||.||... T.xian_3p_3489 1 AAACCGTCGACCGGGAGCTCGG 22	50.0
T.luce_5p_399471		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 .|||.|...||..|.|||...| T.luce_5p_399471 1 CAACTGTCATCGCGTATCCGGT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_252989		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 |.|..||||||.|.|||...|| T.luce_5p_252989 1 AGATTGATCTCGACGATGCGTT 22	63.6
T.luce_3p_617474		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 ||...|||.||.|...|...|| T.luce_3p_617474 1 AAGTTGATTTCGAAATTGATTT 22	50.0
T.luce_5p_481971		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 .||...||.||.|.|...|.|| T.luce_5p_481971 1 TAATGCATTTCGATGTCGTCTT 22	50.0
T.luce_3p_609984		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 |....||||.|||.|.....|| T.luce_3p_609984 1 AGGTCGATCGCAACGCGTGCTT 22	50.0
T.aust_5p_58885		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 |||.|...|.|||||.....|| T.aust_5p_58885 1 AAAAACCCCGCAAGGTCACCTT 22	54.5
T.aust_5p_16396		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 |...|.|.|||||.|...|.|. T.aust_5p_16396 1 AGGAAAACCTCAAAGCGGTTTG 22	50.0
T. luce_5p_678		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 |....|.|.||||||.|.|... T.luce_5p_678 1 ATCATGTTTTCAAGGGTTTCCG 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T. luce_3p_678		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 .|..|.||.|...|.|||.||. T.luce_3p_678 1 CAGTACATGTTCGGTATCGATG 22	50.0
T. aust_5p_3299		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 |.|||.|.||.|......|.|| T.aust_5p_3299 1 ACACACACCTGATTTGGGTTTT 22	50.0
T. xiam_5p_8112		SUT_hvu_mir_000050 1 AAACAGATCTCAAGGATCTATT 22 ...|||...||.|..|.|.||| T.xiam_5p_8112 1 TGCCAGCCATCGAAAACCGATT 22	50.0
T.profu_5p_28449	SUT_hvu_mir_000081	SUT_hvu_mir_000081 1 TCTTCTGAAGCTGTGGAATGTC 22 |..|..|||..||.|.|..||. T.profu_5p_28449 1 TTATGCGAAATTGAGAAGCGTT 22	50.0
T.profu_5p_17011		SUT_hvu_mir_000081 1 TCTTCTGAAGCTGTGGAATGTC 22 |.|..|||.||.....|||.|. T.profu_5p_17011 1 TATGATGATGCGCGACAATTTT 22	50.0
T.profu_5p_30109		SUT_hvu_mir_000081 1 TCTTCTGAAGCTGTGGAATGTC 22 ...||||..||.|||.....|| T.profu_5p_30109 1 CAATCTGTTGCAGTGCCTGATC 22	50.0
T.xian_3p_19710		SUT_hvu_mir_000081 1 TCTTCTGAAGCTGTGGAATGTC 22 ...|| |||||.|.||..|| T.xian_3p_19710 1 GGATC---AGCTGGGTAACATC 19	54.5
T.profu_5p_50983		SUT_hvu_mir_000081 1 TCTTCTGAAGCTGTGGAATGTC 22 |.|...|..||||.|..|.|. T.profu_5p_50983 1 TATGGCGTTGCTGTAGTGTTTG 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.aust_5p_968		SUT_hvu_mir_000081 1 TCTTCTGAAGCTGTGGAATGTC- 22 ..|.|.||. |..|||..|||| T.aust_5p_968 1 GGTCCGGAT-CATTGGGCTGTCG 22	52.2
T. tepi_5p_19555		SUT_hvu_mir_000081 1 TCTTCTGAAGCTGTGGAATGTC 22 ...||||..||.|||.....|| T.tepi_5p_19555 1 CAATCTGTTGCAGTGCCTGATC 22	50.0
T.profu_3p_24901	SUT_hvu_mir_000127	SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 .||.|..|||..|..|.|||| T.profu_3p_24901 1 ATTCCCGCCTGACGATCTTTGT 22	59.1
T.profu_3p_33610		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTT-TGT 22 |.|. |||...||.|||| ||| T.profu_3p_33610 1 CATT-TCCGGATCTTTTTTCTGT 22	65.2
T.profu_3p_33977		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |..|..|..|||.|||..|.|. T.profu_3p_33977 1 CAGGGCCTATTTAATTGGTAGC 22	50.0
T.luce_3p_338996		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |.||.|.|....|..|.|||.| T.luce_3p_338996 1 CCTGTTACTCGCCGGTCTTTTT 22	50.0
T.luce_3p_273440		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 .||..|..|..|.|||||... T.luce_3p_273440 1 TTTATTTTCGATTATTTTTATG 22	50.0
T.xian_3p_16407		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |.|||.|..||.|..|.|...| T.xian_3p_16407 1 CCTGCGCGATTGCGCTATCGAT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_3p_198770		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |.||.|.||.||.....|.|| T.luce_3p_198770 1 CATGTTGCCGTTTCGCGATGGT 22	50.0
T.aust_3p_56497		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |||...|.||||...||....| T.aust_3p_56497 1 CTTCGCCACTTTTCATTGGGAT 22	50.0
T.tepi_3p_6580		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |.||||.||||.|.|....|.| T.tepi_3p_6580 1 CCTGCTGCCTTGCTTCGCGTAT 22	59.1
T.xiam_3p_34788		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |||...||..||..|..||||. T.xiam_3p_34788 1 CTTCTGCCTCTTTTTCGTTTGA 22	54.5
T.tepi_3p_23682		SUT_hvu_mir_000127 1 CTTGCTCC-CTTTCATTTTTTGT 22 .....||. ||||..||||.|| T.tepi_3p_23682 1 GCCCGTCTTCTTTTGTTTTGTG- 22	52.2
T.tepi_3p_11218		SUT_hvu_mir_000127 1 CTTGCTCCCTTTCATTTTTTGT 22 |.|||..||...||.|.|..|. T.tepi_3p_11218 1 CCTGCCTCCGAACAGTCTGGGA 22	50.0
T.profu_5p_49438/ T.tepi_5p_33046	SUT_hvu_mir_000029	SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 ...|.|||.|.|||.|..|.|| T.profu_5p_49438 1 AATCATCGATCCGTTGATCTTC 22	54.5
T.profu_3p_9602/ T.tepi_3p_6592		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 |.|||.|..||..|.||.|... T.profu_3p_9602 1 CTCCGCCTTTTTTTTGTTCGAG 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.profu_5p_45214		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 |||.|..|...|||.|...|.| T.profu_5p_45214 1 CGCGGCGGTGGCGTTGCCGAAC 22	50.0
T.profu_3p_18810		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 |||.|.|||.....|.||..|. T.profu_3p_18810 1 CGCGGCCGCCGGCGCCTATGTG 22	50.0
T.aust_3p_10018		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 ...||||..|.|.|||. ||. T.aust_3p_10018 1 TAACGTCTGTCCTTCGG--ATT 20	50.0
T.luce_3p_471094		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 |||||.|..||....||.|||. T.luce_3p_471094 1 CGCCGCCTTTTTTGTGTGCATA 22	59.1
T.luce_3p_48943		SUT_hvu_mir_000029 1 CGCCGTCGCTT-CGTCGTACATC 22 |||||.|..|| |||.||.. || T.luce_3p_48943 1 CGCCGCCTTTTTCGTGGTTG-TC 22	65.2
T.luce_3p_529459		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 |||..|.|||.|.|.|....|. T.luce_3p_529459 1 CGCTATAGCTGCTTTGCCAGTT 22	50.0
T.xian_3p_3333		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 .|||.||||.|.....|...| T.xian_3p_3333 1 AGCCATCGCTGCCGGTGATCCC 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_282136		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 .||||....||||.|..|...| T.luce_5p_282136 1 TGCCGGTATTTCGGCCGATGAC 22	50.0
T.aust_3p_15365		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 ||||.....|||||...||...	50.0
		T.aust_3p_15365 1 CGCCCGTTTTTCGTTTCACCAG 22	
T.luce_3p_43186		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 ||.....||||..||...|||.	50.0
		T.luce_3p_43186 1 CGGGTCTGCTTTCTCACTCATG 22	
T.luce_3p_585272		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 |||..||||....|.|...|.|	50.0
		T.luce_3p_585272 1 CGCTTTCGCAGGCTTGATGAGC 22	
T.aust_3p_47340		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 ||.||...|...|||.|..||.	50.0
		T.aust_3p_47340 1 CGTCGCATCGGTGTCTTCGATG 22	
T.tepi_3p_8120		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 |.||||.....|.|||.|.|.	50.0
		T.tepi_3p_8120 1 CACCGTTTTGATGCCGTCCTTT 22	
T.aust_5p_22981		SUT_hvu_mir_000029 1 CGCCGTCGCTTCGTCGTACATC 22 .|..|..|||.||.||..||.|	54.5
		T.aust_5p_22981 1 AGTTGAAGCTGCGCCGGCCAGC 22	
T.prof_5p_19418	SUT_hvu_mir_000025	SUT_hvu_mir_000025 1 GTGCT-TATTGACGGTCCAGTGCT 23 |||.| |.|||...|..||...|.	50.0
		T.prof_5p_19418 1 GTGTTCTTTTGGTCGCGCATGCCG 24	

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.profu_3p_21694		SUT_hvu_mir_000025 1 GTGCTTATTGACGGTCCAGTGCT 23 .|.||..||.||.|.| ||...| T.profu_3p_21694 1 CTTCTGCTTTACAGAC-AGAATT 22	52.2
T.xian_3p_20844		SUT_hvu_mir_000029 1 GTGCTTATTGACGGTCCAGTGCT 23 |.|||.||.||..|....||| T.xian_3p_20844 1 GGGCTTTTTTACATTTGGTTGC- 22	56.5
T.luce_3p_556182		SUT_hvu_mir_000029 1 GTGCTTATTGACGGTCCAGTGCT 23 |.|.|| |||.|....||..||. T.luce_3p_556182 1 GGGATT-TTGGCCCGGCATCGCC 22	52.2
T.xian_5p_3333		SUT_hvu_mir_000029 1 GTGCTTATTGACGGTCCAGTGCT 23 .|..|...||||||...| ||| T.xian_5p_3333 1 ATCATCGGTGACGGTGGTG-GCT 22	56.5
T.luce_3p_612357		SUT_hvu_mir_000029 1 GTGCTTATTGACGGTCCAGTGCT 23 |.|.|| ||.|||.||....|.. T.luce_3p_612357 1 GGGGTT-TTTACGTTCTCTGGGG 22	52.2
T.luce_3p_608572		SUT_hvu_mir_000029 1 GTGCTTATTGACGGTCCAGTGCT 23 . ||.|.|..|.||.|.||.||. T.luce_3p_608572 1 C-GCATTTCAAGGGCCAAGGGCA 22	52.2
T.aust_5p_39387		SUT_hvu_mir_000029 1 GTGCTTATTGACGGTCCAGTGCT 23 || |.|.|.|.|.||.|.|.||. T.aust_5p_39387 1 GT-CATTTGGCCTGTGCCGGGCG 22	56.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.aust_3p_37611		SUT_hvu_mir_000029 1 GTGCTTATTGA-CGGTCCAGTGCT 23 .. |.|||| |.|..|.|||. T.aust_3p_37611 1 CG--TCATTGAACAGATCGGTGCG 22	54.2
T.pova_5p_489	SUT_hvu_mir_000095	SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 |||.|||...|.|||.||....	54.5
		T.pova_5p_489 1 TTCATCCAGCCGATGCGCGCGA 22	
T.profu_3p_32260		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 ...|||..||.|.||..|||..	50.0
		T.profu_3p_32260 1 GCGTTCGGAGGATTGCACATGC 22	
T.alka_3p_1023		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 ..|.||.||.|.||||..|...	50.0
		T.alka_3p_1023 1 GACATCACACCGATGGAAAATC 22	
T.luce_5p_31831		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 .||.|..|.|||.||| |...	50.0
		T.luce_5p_31831 1 ATCGTGGCCGCACTGG--AGCC 20	
T.prof_3p_49		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 .||.|. ||| .||||..||	54.5
		T.prof_3p_49 1 CTCCTG--AGC--CGGGCCAAT 18	
T.luce_5p_156594		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 .|..|..|||.|||.|||...	54.5
		T.luce_5p_156594 1 GTTATTGCAGGAATAGGCAATG 22	
T.luce_3p_302533		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 |..|||||....|.|.|...||	50.0
		T.luce_3p_302533 1 TCATTCCCGCGCAGGCGGGAAT 22	

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_65195		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 |||.|.|..|||..|.||.. T.luce_5p_65195 1 TTCCTTCTGGCATGGAGCGG-- 20	50.0
T.luce_5p_354019		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 .||||.||||.|..|.| T.luce_5p_354019 1 ATCTTCAGGCCGGTGAGATTGT 22	50.0
T.aust_3p_7801		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 ..|||||.|.|||...|....| T.aust_3p_7801 1 GACTTCCGATCAAAAAGTGCGT 22	50.0
T.tepi_3p_20938		SUT_hvu_mir_000029 1 TTCTTCCCAGCAATGGGCATAT 22 ...|||..||.|.||..|||.. T.tepi_3p_20938 1 GCGTTCGGAGGATTGCACATGC 22	50.0
T.pova_3p_2290	SUT_hvu_mir_000026	SUT_hvu_mir_000026 1 GAGCTTATTGACGGTCCAGTGCT 23 |..||.||||...|||.| |.. T.pova_3p_2290 1 GGTCTGATTGATTTTCCCG-GTC 22	56.5
T.luce_3p_464921		SUT_hvu_mir_000026 1 GAGCTTATTGACGGTCCAGTGCT 23 ||....|.|.|||||..|.. | T.luce_3p_464921 1 GATGACAATTACGGTCGGGAT-T 22	52.2
T.xian_3p_20844		SUT_hvu_mir_000026 1 GAGCTTATTGACGGTCCAGTGCT 23 |.|||.||.||..|....||| T.xian_3p_20844 1 GGGCTTTTTTACATTTGGTTGC- 22	56.5
T.xian_5p_22881		SUT_hvu_mir_000026 1 GAGCTTATTGACGGTCCAGTGCT 23 .|.|.|..|||.| |||.... T.xian_5p_22881 1 CACCATCCTGACCG-CCAGCATG 22	52.2

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_3p_556182		SUT_hvu_mir_000026 1 GAGCTTATTGACGGTCCAGTGCT 23 |.|.|| |||.|....||..||. T.luce_3p_556182 1 GGGATT-TTGGCCCGGCATCGCC 22	52.2
T.aust_5p_57006		SUT_hvu_mir_000026 1 GAGCTTATTGACGGTCCA-GTGCT 23 |...|..|||.|||.|.| ||| T.aust_5p_57006 1 GTTTTGCTTGTCGGGCAACGTG-- 22	54.2
T.xian_3p_32022	SUT_hvu_mir_000158	SUT_hvu_mir_000158 1 ACATGCATCGTGCTGGGGAGAAAA 24 |||.|||.|||.|||..| T.xian_3p_32022 1 ------ATCATGCCGGGCAGATCA 18	54.2
T.aust_3p_56957		SUT_hvu_mir_000158 1 ACATGCATCGTGCTGGGGAGAAAA 24 |.|..||.|.||.|||.|..|. T.aust_3p_56957 1 AGACCCAGCTTGGTGGCGGCAT-- 22	50.0
T.luce_5p_23450		SUT_hvu_mir_000158 1 ACATGCATCGTGCTGGGGAGAAAA 24 |..|||.|..|| |.|.|||... T.luce_5p_23450 1 ATGTGCCTGTTG--GCGCAGATCG 22	50.0
T.luce_3p_531055		SUT_hvu_mir_000158 1 ACATGCATCGTGCTGGGGAGAAAA 24 |..||. ||.||..|||.||..| T.luce_3p_531055 1 AACTGG--CGGGCGCGGGTGAGGA 22	54.2
T.tepi_3p_28590		SUT_hvu_mir_000158 1 ACATGCATCGTGCTGGGGAGAAAA 24 . ||||.|..||.....||.||| T.tepi_3p_28590 1 T-ATGCGTTTTGTCCATGACAAA- 22	50.0
T.profu_5p_43344	SUT_hvu_mir_000084	SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 ..|||..|.|..||.|||.|.. T.profu_5p_43344 1 ATCTGTTTTGGGGCGATCCGAA 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.meso_3p_3026		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 .....|||||||.|.|||.| T.meso_3p_3026 1 CTTGGCGTCGAAGGCATGA--- 19	54.5
T.profu_5p_44486		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 .|..||.|.||.||.|.|.|.. T.profu_5p_44486 1 GGACGCATTGATGCGACCCGAG 22	50.0
T.profu_3p_24324		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 ||..|||..|..|||...|.|. T.profu_3p_24324 1 TGGCGCGCTGGAGCCCGGAACA 22	50.0
T.profu_5p_18148		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 |||.||..||||.....|.|.. T.profu_5p_18148 1 TGCCGCCGCGACCATCGCGGGG 22	50.0
T.xian_5p_17518		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCA-GCC 22 .||||.|.|| ||.|||.. ||| T.xian_5p_17518 1 AGCTGTGCCG-CGTCATGTTGCC 22	65.2
T.tepi_5p_8405		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 ..|.||||.|.|||..|...|. T.tepi_5p_8405 1 GTCGGCGTTGTCGCGCTGTTCA 22	50.0
T.profu_5p_12074		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 ..|.|.|.||...||..|.||| T.profu_5p_12074 1 ATCGGGGCCGGATCCGGCGGCC 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.profu_3p_56542		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 |||.|.|....|||...|.|.| T.profu_3p_56542 1 TGCCGAGGGCCCGCGTGCGGTC 22	50.0
T.aust_3p_11614		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 ..|.|...|..||..||.||| T.aust_3p_11614 1 GACAGTTCCAGCGTTATTAGCC 22	50.0
T.aust_5p_40281		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCA-TCAGCC- 22 |||||||..|..|| |.|.|| T.aust_5p_40281 1 --CTGCGTCTCCTGCAGTAATCCG 22	58.3
T.tepi_3p_30879		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC-- 22 || |||||..|||...|||. T.tepi_3p_30879 1 TG--GCGTCGTTGCCGAAAGCTGG 22	58.3
T.aust_5p_53204		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCA--GCC 22 ||.||. |||..||||| |.. T.aust_5p_53204 1 TGATGG--CGATACCATCAAGGGG 22	58.3
T.aust_5p_28718		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGC--C 22 || ||.|||.||....|.|| | T.aust_5p_28718 1 TG--GCATCGGCGGTCGCGGCATC 22	54.2
T.tepi_3p_3446		SUT_hvu_mir_000084 1 --TGCTGCGTCGACGCCATCAGCC 22 |||.||.|||||...|||| | T.tepi_3p_3446 1 AGTGCGGCATCGACCTGATCA--C 22	62.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.tepi_3p_33254		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 |.|.|.||...||.|.|||... T.tepi_3p_33254 1 TTCGGTGTTTGCGACTTCAAAG 22	50.0
T.tepi_5p_7722		SUT_hvu_mir_000084 1 TGCTGCGTCGACGCCATCAGCC 22 ||..|.||..||.||...||. T.tepi_5p_7722 1 -GCGACATCATCGGCACTTGCG 21	50.0
T.alka_5p_4684	SUT_hvu_mir_000112	SUT_hvu_mir_000112 1 CCTACTAACG-CGTTTCCTTTCCA 23 .||...|.|| |||||..| |. T.alka_5p_4684 1 TCTGGCATCGGCGTTTCTAT--CG 22	54.2
T.xiam_3p_34788 T.tepi_3p_18168		SUT_hvu_mir_000112 1 CCTACTAACGCGTTTCCTTTCCA 23 | |.||..|.|.|||.|.||..| T.xiam_3p_34788 1 C-TTCTGCCTCTTTTTCGTTTGA 22	56.5
		SUT_hvu_mir_000112 1 CCTACTAACGCGTTTCCTTTCCA 23 ..| |...|.|.||..|||||. T.tepi_3p_18168 1 GGT-CGGTCTCCTTGGCTTTCCT 22	52.2
T.profu_3p_32508	SUT_hvu_mir_000027	SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 |||..|..|..|..||||.|| T.profu_3p_32508 1 --GGGTGTTTTTTTGCTATAGTTC 22	54.2
T.pova_5p_390		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 .|.|.|...|| |.||.|..|.|| T.pova_5p_390 1 CTTGAAGACCT-GCATCAGCGTTC 23	50.0
T. xian_3p_6822		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 |.|||.||..|. |||....|.| T.xian_3p_6822 1 AGGGGTTTTTTA--ATTGGTAGCC 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.xian_3p_18366		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 ...||.|| |||..||.|.||.. T.xian_3p_18366 1 GGCGGCTT--TCGCGTTTTGGGGG 22	50.0
T.aust_3p_54777		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 ..|||.| .|..|.|||..||. T.aust_3p_54777 1 CGGGGCT--GTTTTTTTATCTGTG 22	50.0
T.aust_3p_58885		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 |||.||..||.|.|.||.|.|. T.aust_3p_58885 1 --GGGGTTTTTCTTTTAATTGATG 22	54.2
T. luce_3p_474240		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 ..|||.|| |.|..|||..||.. T.luce_3p_474240 1 TGGGGGTT--TTGCTTTAGGGGCG 22	50.0
T.aust_5p_39756		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 |.|..|.| || |.|.|..||.|| T.aust_5p_39756 1 AGGTTAAT-CT-GGACTTCAGCTC 22	54.2
T.luce_3p_559495		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTAT-TATAGGTC 24 |.|||.|.||.|.|.. ||||. T.luce_3p_559495 1 ACGGGTTCGCGCATTCCTATAA--- 22	52.0
T.luce_3p_421879		SUT_hvu_mir_000027 1 ATG-GGATTGCTCGTATTATAGGTC 24 |.. ||.|||.|. |||||||. T.luce_3p_421879 1 AACTGGCTTGTTT---TTATAGGTT 22	60.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.tepi_5p_11782		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 ||.|||.|.|..|..|||.|.. T.tepi_5p_11782 1 -TGTGATGGTTTCTTCTATCGCA- 22	50.0
T.tepi_3p_22942		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 ||||.|.|||..|.||.|..|. T.tepi_3p_22942 1 -TGGGGTGGCTTTTCTTTTTCGG- 22	54.2
T.xiam_3p_6543		SUT_hvu_mir_000027 1 ATGGGATTGCTCGTATTATAGGTC 24 | |.||||..||.||.|..|.. T.xiam_3p_6543 1 A--GAATTGCGGGTCTTTTTTGGT 22	54.2
T.profu_3p_38494	SUT_hvu_mir_000142	SUT_hvu_mir_000142 1 CTATGTAGACTT--TTGTTTAAA 21 ..||||.|.|.. ||||..| T.profu_3p_38494 1 ACATGTGGTCAAGGTTGTTCGA- 22	52.2
T.profu_5p_51572		SUT_hvu_mir_000142 1 CTATGTAGACT-TTTGTTTAAA 21 ..|.|.|||.. |||.|.|.|. T.profu_5p_51572 1 TGACGCAGAGGCTTTCTCTCAT 22	50.0
T.luce_3p_599500		SUT_hvu_mir_000142 1 CTATGTA-GACTTTTGTTTAAA 21 ..|.|.. |.|||..|||.|| T.luce_3p_599500 1 ACAGGCCTGCCTTACGTTTTAA 22	54.5
T.alka_3p_3811	SUT_hvu_mir_000046	SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 ......|||.|.||.|..||| T.alka_3p_3811 1 CCGGGTTTGACCGGATGAAGGC 22	50.0
T.profu_3p_20856		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 .||...|.|.|.||||...||| T.profu_3p_20856 1 TGATGTTGGTCGGGCTGATGGC 22	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.xian_3p_19710		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 |||.| .||||.|.....| T.xian_3p_19710 1 GGATC---AGCTGGGTAACATC 19	50.0
T.aust_5p_13346		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 .||||. |||...|.||| T.aust_5p_13346 1 CGAACT----CTGCACCAAGGC 18	54.5
T.luce_3p_601689		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 ||..||||...|.|..|..|.| T.luce_3p_601689 1 GGGGCGTTTTTTTGTGCCGGTC 22	50.0
T.luce_3p_556182		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 ||.|..||||..|.....|.| T.luce_3p_556182 1 GGGATTTTGGCCCGGCATCGCC 22	50.0
T.luce_5p_385818		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 ..||.|.|.|||.|..||...| T.luce_5p_385818 1 CAAAGGATTGCTTGGCCGCCCC 22	50.0
T.xian_5p_20844		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 .|.|..||..|.|...||.||| T.xian_5p_20844 1 CGCAGCTTTCCGGCTGCGGGGC 22	50.0
T.luce_5p_593263		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 .|...|.||..|.|||..||| T.luce_5p_593263 1 AGGCTGCTGCTTCGCTCCGGGC 22	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_3p_354019		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 |.....|||...||.|||.|| T.luce_3p_354019 1 GCCGGTTTGCTGGGGTCGGGGC 22	54.5
T.aust_5p_5027		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 .||...||.|||||.|..... T.aust_5p_5027 1 CGATTATTCGCTGGCGCTTTTT 22	50.0
T.aust_5p_32539		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 .|..||..|||.|..|.|.||. T.aust_5p_32539 1 AGGGCGAAGGCAGCGTTGCGGA 22	50.0
T.xiam_5p_35854		SUT_hvu_mir_000046 1 GGAACGTTGGCTGGCTCGAGGC 22 .|..||.....|.||||.|| T.xiam_5p_35854 1 AGTGCGGATCGCGCCTCGATGC 22	50.0
T.alka_5p_1519	SUT_hvu_mir_000023	SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 ..|||....|||..||.||..| T.alka_5p_1519 1 AAGGGTTGGTTCCGACCTATCT 22	50.0
T.alka_5p_5116		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 .||..|||..|.||.|||.... T.alka_5p_5161 1 TTGAAAGATATATATCTTCCTG 22	50.0
T.luce_5p_210055		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 ..||..||.||.|.||...|.| T.luce_5p_210055 1 TCGGCGGATTTTTGACCCGATT 22	50.0
T. aust_3p_20162		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 ..|...|||||||.|.....| T.aust_3p_20162 1 TGGCATGACTTCTAGCCCTGGT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.aust_3p_56497		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 ||..|..||||.|.|.|...|| T.aust_3p_56497 1 CTTCGCCACTTTTCATTGGGAT 22	54.5
T.luce_3p_44717		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 .|||..|.||..|.||.|...| T.luce_3p_44717 1 ATGGTTGCCTGATGACGTGCTT 22	50.0
T.tepi_3p_9218		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 .|...||..||.|..||.|.|| T.tepi_3p_9218 1 TTCCTAGTTTTTTCTCTCATAT 22	50.0
T.tepi_3p_33254		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT 22 .|.||.|..|.|.|..|.|||. T.tepi_3p_33254 1 TTCGGTGTTTGCGACTTCAAAG 22	50.0
T.aust_3p_968		SUT_hvu_mir_000023 1 CTGGGAGACTTCTAACTTAAAT- 22 .||. |||.|||.|.|..||.. T.aust_3p_968 1 ATGC-AGATTTCAACCCGAACCA 22	52.2
T.pova_5p_489	SUT_hvu_mir_000094	SUT_hvu_mir_000094 1 TCCATCCATCCGATCCCAGGAG 22 |.||||||.|||||.|..|... T.pova_5p_489 1 TTCATCCAGCCGATGCGCGCGA 22	63.5
T.profu_5p_57479		SUT_hvu_mir_000094 1 TCCATCCATCCGATCCCAGGAG 22 |.|.||.||||....|.||||. T.profu_5p_57479 1 TACCTCGATCCATATCGAGGAA 22	59.1
T.profu_3p_12216		SUT_hvu_mir_000094 1 TCCATCCATCCGATCCCAGGAG 22 |.||...|.|||.|.|..|..| T.profu_3p_12216 1 TTCAAGGAGCCGCTGCATGTTG 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.xian_5p_17681		SUT_hvu_mir_000094 1 TCCATCCATCCGATCCCAGGAG 22 ....||||||.|.|.|..|..| T.xian_5p_17681 1 CTATTCCATCGGTTACATGTCG 22	50.0
T.aust_5p_39387		SUT_hvu_mir_000094 1 TCCATCCATCCGATCCCAGGAG 22 ..|||....||..|.||.||.| T.aust_5p_39387 1 GTCATTTGGCCTGTGCCGGGCG 22	50.0
T.luce_5p_585272		SUT_hvu_mir_000094 1 TCCATCCATCCGATCCCAGGAG 22 |...|.||||...|.|..||.| T.luce_5p_585272 1 TGAGTTCATCAAGTGCGCGGCG 22	50.0
T.alka_5p_4942/ T.luce_5p_520914	SUT_hvu_mir_000039	SUT_hvu_mir_000039 1 TGAATTTGTTTAACTAGAATTTAT 24 |.|||..|. |.|.|..|.||| T.alka_5p_4942 1 TTAATCCGG--ACCCATTAATTAT 22	54.2
T.profu_3p_56451		SUT_hvu_mir_000039 1 TGAATTTGTTTAACTAGAATTTAT 24 | ||.|.|||.|...|||.|.|| T.profu_3p_56451 1 T--ATCTTTTTCAAAGGAAGTCAT 22	58.3
T.profu_3p_50983		SUT_hvu_mir_000039 1 TGAATTTGTTTAACTAGAATTTAT 24 | |.|..||..|||.|...||| T.profu_3p_50983 1 T--AGTGTTTGCACTGGTGGTTAT 22	54.2
T.xian_5p_9958		SUT_hvu_mir_000039 1 TGAATTTGTTTAACTAGAATTTAT 24 ..|.||.|| |||.|.|.||... T.xian_5p_9958 1 ATATTTCGT--AACCATATTTACG 22	50.0
T.aust_3p_3291		SUT_hvu_mir_0000039 1 TGAATTTGTTTAACTAGAATTTAT 24 |.||||..|..||...|.|.| T.aust_3p_3291 1 --AGTTTGTAAATATAACGTCTGT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.aust_5p_47340		SUT_hvu_mir_000039 1 TGAATTTGTTTAACTAGAATTTAT 24 |||..||||| .|.|.||....| T.aust_5p_47340 1 TGATGTTGTT--GCCAAAAAGCTT 22	54.2
T.tepi_5p_33271		SUT_hvu_mir_000039 1 TGAATTTGTTTAACTAGAATTTAT 24 ||..|||.|.|.|| |..|||.. T.tepi_5p_33271 1 TGTCTTTTTCTGAC--GTTTTTTC 22	54.2
T.profu_3p_14407	SUT_hvu_mir_000034	SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 |||...|.|||..|||..|... T.profu_3p_14407 1 AGCGTGTCATGGCCTTCATGCC 22	50.0
T.alka_5p_3838		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 .|.||..|||.|.|.|....|| T.alka_5p_3838 1 CGAAACGGATCACCGTTTCAGG 22	50.0
T.profu_5p_24324		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 .|....|..|||||..|.||. T.profu_5p_24324 1 CGTCGGTCGTGAGCTGGCGCGC 22	50.0
T.luce_5p_82704		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 |..|....|.|.||.|.||.| T.luce_5p_82704 1 AATATCGCAAGGGCGTTCTGGG 22	50.0
T.luce_3p_82704		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 .||.|..||.|..|||.|...| T.luce_3p_82704 1 TGCCATCGAAGCCCTTTCGGTG 22	50.0
T.profu_5p_21694		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTC-GG 22 ||||||.|.||. ||.|.|. || T.profu_5p_21694 1 AGCAAAAGCTGC-CTAATTAAGG 22	65.2

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T. aust_3p_23133		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 |....|.||||...|..||.| T.aust_3p_23133 1 ACTGTAAGATGAAGATCATCCG 22	50.0
T.luce_5p_246051		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 |.||..|.|||...||...||. T.luce_5p_246051 1 ATCAGGTCATGCAATTTGGCGA 22	50.0
T.aust_3p_36180		SUT_hvu_mir_000034 1 AGCAAATGATGAGCTTACTCGG 22 |.|..|...|.|.|||.||.. T.aust_3p_36180 1 AACCCAGTTTAATCTTAGTCAT 22	50.0
T.alka_5p_3811	SUT_hvu_mir_000228	SUT_hvu_mir_000228 1 TTTGCCATCAGCCTTGGG-GCT 21 ..||..|||.||||.|. |.. T.alka_5p_3811 1 GCTGGTATCGGCCTTTGCCGGG 22	54.5
T.alka_5p_4684		SUT_hvu_mir_000228 1 TTTGCCATCAGCCTTGGGG-CT 21 |.||.|||.||.||.... |. T.alka_5p_4684 1 TCTGGCATCGGCGTTTCTATCG 22	54.5
T.profu_3p_51013		SUT_hvu_mir_000228 1 TTTGCCATCAGCCTTGGGGC--T 21 .|.||..|| |.||||.|. | T.profu_3p_51013 1 ATCGCTTTC-GGCTTGGTGTAGT 22	56.5
T.profu_3p_28449		SUT_hvu_mir_000228 1 TTTGCCATCAGCCTTGGGGC-T 21 .||....|||.|.|.||.| | T.profu_3p_28449 1 ATTTGTTTCAGGCATAGGACAT 22	54.5
T.profu_3p_54785		SUT_hvu_mir_000228 1 TTTGCCATCAGCCTTGGG-GCT 21 ||..|.|.|.||.....| ||| T.profu_3p_54785 1 TTGCCGAACGGCTGAAAGAGCT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.profu_5p_24235		SUT_hvu_mir_000228 1 TTTGCCATCAG-CCTTGGGGCT 21 |.|||||.||. ||....||.| T.profu_5p_24235 1 TATGCCAACAATCCGACCGGGT 22	59.1
T.xian_3p_3512		SUT_hvu_mir_000228 1 TTTGCCATCA-GCCTTGGGGCT 21 .||..|.|.. ||.|||.|||. T.xian_3p_3511 1 ATTCACTTTTTGCATTGCGGCA 22	54.5
T. aust_5p_2750		SUT_hvu_mir_000228 1 TTTGCCATCA-GCCTTGGGGCT 21 .||||...|| ||.|.|.|..| T.aust_5p_2750 1 ATTGCGTGCAAGCATCGCGATT 22	54.5
T.aust_5p_17456		SUT_hvu_mir_000228 1 TTTGCCATCAGCCTTGG-GGCT 21 .|.|.|.||.|.||.|. ||.| T.aust_5p_17456 1 CTGGGCTTCTGGCTCGTTGGTT 22	54.5
T.aust_5p_7549		SUT_hvu_mir_000228 1 TTTGCCATCAGCCTTGGGGCT- 21 .|||.|||||.|..|||.... T.aust_5p_7549 1 CTTGGCATCACCGATGGCAAGA 22	54.5
T.profu_3p_49438/ T.tepi_3p_33046	SUT_hvu_mir_000079	SUT_hvu_mir_000079 1 AAGTTGG-GCAATAATGTTGTA 21 ||.||.| |||....|.|.||. T.profu_3p_49438 1 AACTTTGTGCAGCTGTTTGGTC 22	54.5
T.alka_5p_333		SUT_hvu_mir_000079 1 AAGTTGGG-CAATAATGTTGTA 21 ..|||... ||||.||...||. T.alka_5p_333 1 TTGTTACTTCAATGATCCGGTT 22	50.0
T.alka_3p_4120		SUT_hvu_mir_000079 1 AAGTTGG-GCAATAATGTTGTA 21 ||..... ||.|.||||||.|. T.alka_3p_4120 1 AAAACCCCGCCAGAATGTTCTG 22	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.profu_3p_38494		SUT_hvu_mir_000079 1 AAGT-TGGGCAATAATGTTGTA 21 |..| |||.|||...||||..| T.profu_3p_38494 1 ACATGTGGTCAAGGTTGTTCGA 22	59.1
T.luce_5p_543369		SUT_hvu_mir_000079 1 AAGTTGG--GCAATAATGTTGTA 21 |||...| ||| |.|||.||.. T.luce_5p_543369 1 AAGGCCGCAGCA-TGATGCTGCG 22	56.5
T.luce_3p_285535		SUT_hvu_mir_000079 1 A--AGTTGGGCAATAATGTTGTA 21 | ||. |||.|||||||...|. T.luce_3p_285535 1 ATCAGC-GGGAAATAATGAATTG 22	60.9
T.luce_3p_406605		SUT_hvu_mir_000079 1 AAGTTGGGCAATAATGTTGTA- 21 .||||.|.|....||.|||.. T.luce_3p_406605 1 CAGTTCGACCGACATTTTGGCG 22	50.0
T.aust_3p_63319		SUT_hvu_mir_000079 1 AAGT-TGGGCAATAATGTTGTA 21 |||. |....|.|||||..|. T.aust_3p_63319 1 AAGCCTCATGATTAATGTGTTC 22	54.5
T.aust_3p_53294		SUT_hvu_mir_000079 1 AAGTTGGGCAATAATGTTGTA 21 .....|||||||...|.|||. T.aust_3p_53294 1 TCAAGGGGCAATGGCGCTGTT 21	52.4
T.xian_5p_9480	SUT_hvu_mir_000180	SUT_hvu_mir_000180 1 CTTTCTGAA-CTCTTCTATTCCAGG 24 .||...||| ||.||||....|||. T.xian_5p_9480 1 ATTCAGGAATCTGTTCTGACGCAGC 25	56.0
T.profu_3p_5000		SUT_hvu_mir_000180 1 CTTTCTGAACTCTTCTATTCCAGG 24 ..|...||||.| .|||.||.. T.profu_3p_5000 1 GGTAACGAACTGT--AATTACACT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_285535		SUT_hvu_mir_000180 1 CTTTCTGAACTCTTCTATTCCAGG 24 .||...|||.||.|| |||. T.luce_5p_285535 1 TTTGAGGAATTCCTCGAT--CAGC 22	58.3
T.aust_3p_49513		SUT_hvu_mir_000180 1 CTTTCTGAACTCTTCTATTCCAGG 24 |..|.|.|||..|...| |||.| T.aust_3p_49513 1 CAATTTAAACATTGTCA--CCATG 22	50.0
T.tepi_3p_22942		SUT_hvu_mir_000180 1 CTTTCTGAACTCTTCTATTCCAGG 24 .....||. ||.|||.||.| || T.tepi_3p_22942 1 TGGGGTGG-CTTTTCTTTTTC-GG 22	54.2
T.luce_3p_14713	SUT_hvu_mir_000041	SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 ||.|||. |.|....|||..| T.luce_3p_14713 1 TTTGCGC-GATGGGTCCCTGAT- 21	52.2
T.profu_3p_41148		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 |||..||...|.|||...|.|| T.profu_3p_41148 1 TTGCGGGTTTTGCTGTGATGTT- 22	52.2
T.luce_5p_48943		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 ||.||||.| ||||..|.....| T.luce_5p_48943 1 TTTGCGGCG-TCCTTTCAATCGT 22	56.5
T.luce_5p_210055		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 |.|||||..|. ||.||...|| T.luce_5p_210055 1 TCGGCGGATTTT-TGACCCGATT 22	60.9
T.luce_3p_559608		SUT_hvu_mir_000041 1 TT-GGCGGAGCTCCTGCCCTATTT 23 || ||||..| |||.|.|...|| T.luce_3p_559608 1 TTTGGCGTTG--CCTACGCCTATT 22	58.3

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.xian_5p_16719		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 .|...|||.. |||..|.|.||| T.xian_5p_16719 1 ATTTTGGATG-CCTTGCGTGTTT 22	52.2
T.luce_5p_637434		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 ||.. |..||.|||.|...||| T.luce_5p_637434 1 TTTT-GTGGCACCTGACAACTTT 22	56.5
T.luce_3p_385818		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 |||||.|. |.|.....|||..| T.luce_3p_385818 1 TTGGCCGC-CCCGAAAGCTACCT 22	52.2
T.luce_3p_8020		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 . |||.|.|||..|||..|.||. T.luce_3p_8020 1 C-GGCCGGGCTTTTGCTGTTTTG 22	56.5
T.luce_3p_132929		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 |||.||..|||..|.|||.|.. T.luce_3p_132929 1 TTGCCGTTGCTGGTCCCCGAAG- 22	56.5
T.aust_3p_57940		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 |..||. ||||..||.|||.||. T.aust_3p_57940 1 TGAGCC-AGCTAGTGTCCTTTTG 22	60.9
T.xiam_5p_37696		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 |.....||.||..||||||| T.xiam_5p_37696 1 TCATTTTTGCGCCGACCCTATT- 22	52.2

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.tepi_3p_8405		SUT_hvu_mir_000041 1 TTGGCGGAGCTCCTGCCCTATTT 23 ||...|||||..|||| |.||. T.tepi_3p_8405 1 TTCAAGGAGCCGCTGCA-TGTTG 22	60.9
T.profu_5p_24901	SUT_hvu_mir_000135	SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 ...|.||.|.||.|.|..|.|| T.profu_5p_24901 1 CCGAAGAGGCCCAGGTGCGGTT 22	50.0
T.profu_5p_15172		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 |||.||.|..|.|.....|.|| T.profu_5p_15172 1 ATTTTGCAATCGCTTATCGCTT 22	50.0
T.xian_3p_20546		SUT_hvu_mir_000135 1 ATTATGAAGACCCGAT--CGGATT 22 .||.....|..|||.| |||||. T.xian_3p_20546 1 CTTGCACCGGGCCGCTTTCGGATG 24	50.0
T.xian_3p_32022		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 ||.|||..|..|.||||. T.xian_3p_32022 1 ATCATGCCGGGCAGATCA---- 18	50.0
T.profu_3p_21694/ T.tepi_3p_13698		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 .||.||.....|.||..|.||| T.profu_3p_21694 1 CTTCTGCTTTACAGACAGAATT 22	50.0
T.xian_5p_16719		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 |||.||.|..||.....|.|| T.xian_5p_16719 1 ATTTTGGATGCCTTGCGTGTTT 22	50.0
T.aust_5p_36180		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 .|.||.|.||...|..|||.|| T.aust_5p_36180 1 CTGATTAGGAGTTGGCCGGGTT 22	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_316125		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 ..|.||.|||.|..|.||... T.luce_5p_316125 1 GATGTGGAGAACCTGTTGGTCG 22	50.0
T.luce_5p_394944		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 ...|..|.||.|||.|||... T.luce_5p_394944 1 CCCAGCATGATCCGTTCGGCGG 22	50.0
T.luce_5p_465632		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 |.|..|||...|.|.|||... T.luce_5p_465632 1 AATCGGAAGCGACAAACGGCGC 22	50.0
T.luce_5p_235876		SUT_hvu_mir_000135 1 ATTATGAAGACCCGATCGGATT 22 |.|||..|..|||..|....| T.luce_5p_235876 1 AATATTTATGCCCGGCCTCGAT 22	50.0
T.luce_3p_520914	SUT_hvu_mir_000062	SUT_hvu_mir_000062 1 GCGAACGAACGA--TCTAAACT 20 ...||..||.|. ||..|||| T.luce_3p_520914 1 CATAATTAATGTGTTCGGAACT 22	50.0
T.profu_3p_5000		SUT_hvu_mir_000062 1 GCGAACGAACGATC--TAAACT 20 |..||||||..|. ||.||| T.profu_3p_5000 1 GGTAACGAACTGTAATTACACT 22	63.6
T.luce_3p_282136		SUT_hvu_mir_000062 1 GCGAAC--GAACGATCTAAACT 20 |...|| |||.|...|.||.| T.luce_3p_282136 1 GATGACCTGAAGGTCTTCAAAT 22	50.0
T.tepi_3p_20938		SUT_hvu_mir_000062 1 GCGAACGAACGATCTAAACT-- 20 |||..||.|.|||...|..| T.tepi_3p_20938 1 GCGTTCGGAGGATTGCACATGC 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.profu_3p_33954	SUT_hvu_mir_000186	SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ...|.||.|.|||||..|.. T.profu_3p_33954 1 GTCCACTTCCGCTGCGCCGTTT 22	54.5
T. alka_3p _2265		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |.|.|||.|||.|.| T.alka_3p_2265 1 TTGAGCTTCTGATACCGTATGC 22	59.1
T.profu_3p_37885/ T.tepi_3p_24769		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ||.|.|....|.||...|||.| T.profu_3p_37885 1 TGCCACCGGCGTTGTTGTCTTC 22	50.0
T.profu_3p_7879/ T.tepi_3p_5471		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ||.||...|.||||..||.... T.profu_3p_7879 1 TGTCGAGGCGGCTGGTCTGCGT 22	50.0
T.profu_5p_49903		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 .|..||.||....|..|||.|| T.profu_5p_49903 1 CGAGGCCCCGATGGGCCTCCCC 22	50.0
T.profu_5p_18810		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 .||||.||.||.|...|.|..| T.profu_5p_18810 1 CGGCGATCATGATTGCCGCGGC 22	54.5
T.tepi_5p_16607		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |. |....|||.|||.|.|| T.tepi_5p_16607 1 TT-CAAGTCTGATGCCCGCGCC 21	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.aust_5p_26441		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ....||.. ||||.|||| T.aust_5p_26441 1 CTTGGCAG--GCTGGGCGCTCC 20	54.5
T.tepi_5p_11638		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |.. .||.|.|||.|||..|. T.tepi_5p_11638 1 TTT--GTCGTTCTGGGCTGGCA 20	50.0
T.meso_3p_3086		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ..||||.| || ||| ||..|| T.meso_3p_3086 1 CTGCGCGC-TG-TGC-CTTGCC 19	63.6
T.luce_5p_56287		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |.|||.|.|||..|..|||.|| T.luce_5p_56287 1 TTGCGGTTCTGTCGAACTCGCC 22	63.6
T.luce_5p_389406/ T.luce_5p_389408		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |.|.||..|.|||..||....| T.luce_5p_389406 1 TCGTGCGTCAGCTTGGCGTGAC 22	50.0
T.xian_3p_22099		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |||.|.....|||||...|. T.xian_3p_22099 1 TGGTGTCGGGCCTGCGCGAACA 22	50.0
T.luce_5p_522493		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 .|...||..|.|||.|.||| T.luce_5p_522493 1 CGTGCCTTATACTGCACGCTCC 22	59.1

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_181596		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ||.|||.||.|..|.||.|..| T.luce_5p_181596 1 TGTCGCGCCCGGCGTGCCCGGC 22	59.1
T.luce_3p_246818		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ||.|||...||.|||.....|. T.luce_3p_246818 1 TGCCGCCGGTGTTGCTGCGGCA 22	50.0
T.aust_3p_39387		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ||.||..|.|.|.||.||..|. T.aust_3p_39387 1 TGCCGGGCGTTCCGCACTTGCA 22	54.5
T.luce_5p_531055		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |.|||||...|.|.|||..... T.luce_5p_531055 1 TTGCGCTATCGATTCGCAACTG 22	50.0
T.tepi_3p_30879		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 |||||....|||.|....||.. T.tepi_3p_30879 1 TGGCGTCGTTGCCGAAAGCTGG 22	50.0
T.aust_3p_7549		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ||||.....|||.||..||.|. T.aust_3p_7549 1 TGGCAAGATTGCCGCCATCGCG 22	54.5
T.aust_3p_34002		SUT_hvu_mir_000186 1 TGGCGCTCCTGCTGCGCTCTCC 22 ...|.|||||||.||.|..... T.aust_3p_34002 1 CTTCTCTCCTGCAGCTCGTGAT 22	50.0
T.alka_5p_333	SUT_hvu_mir_000061	SUT_hvu_mir_000061 1 TAGGTTCATCCGTTGTC--GCT 20 |.|.|.|.||..|..|| |.| T.alka_5p_333 1 TTGTTACTTCAATGATCCGGTT 22	50.0

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.pova_5p_489		SUT_hvu_mir_000061 1 TAGGTTCATCCGTTGT-CGCT- 20 |...|.||.|||.||. |||. T.pova_5p_489 1 TTCATCCAGCCGATGCGCGCGA 22	54.5
T.luce_5p_39407		SUT_hvu_mir_000061 1 TAGGTTCA-TCC-GTTGTCGCT 20 ..||..|. ||| |||||..|. T.luce_5p_39407 1 CCGGAACGGTCCAGTTGTTCCG 22	54.5
T.xian_5p_19488		SUT_hvu_mir_000061 1 TAGGTTCATCCGTTGTCG--CT 20 |.||||..|.|.|||.|| || T.xian_5p_19488 1 TCGGTTTCTGCCTTGGCGGGCT 22	63.6
T.luce_5p_245911		SUT_hvu_mir_000061 1 TAGGTTCATCCGTTGTC--GCT 20 |.||..||...||..|| |.| T.luce_5p_245911 1 TGGGGCCAAATGTGATCTTGGT 22	50.0
T.aust_5p_56957		SUT_hvu_mir_000061 1 TAGGTTCATCC--GTTGTCGCT 20 |...|.|||| |..|.||.. T.aust_5p_56957 1 TGATTCCATCCTGGCGGCCGTA 22	50.0
T.luce_5p_235876		SUT_hvu_mir_000061 1 TAGGTTCAT--CCGTTGTCGCT 20 .|..||.|| |||...|||.| T.luce_5p_235876 1 AATATTTATGCCCGGCCTCGAT 22	54.5
T.luce_5p_585272		SUT_hvu_mir_000061 1 TAGGTTCATCCGTTGTC--GCT 20 |..|||||||...||.. ||. T.luce_5p_585272 1 TGAGTTCATCAAGTGCGCGGCG 22	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_5p_502941		SUT_hvu_mir_000061 1 TAGGTTCATCCG--TTGTCGCT 20 ||.||..||||| |.||..|. T.luce_5p_502941 1 TATGTCGATCCGGATCGTATCC 22	59.1
T.alka_3p_3149	SUT_hvu_mir_000043	SUT_hvu_mir_000043 1 GGCGGATGTAGCCA-AGTTGAG 21 ||||..|...|||. |.|.|.| T.alka_3p_3149 1 GGCGCTTCGGGCCGGAATGGCG 22	54.5
T.profu_5p_44486		SUT_hvu_mir_000043 1 GG-CGGATGTAGCCAAGTTGAG 21 || ||.||..|..|.|...||| T.profu_5p_44486 1 GGACGCATTGATGCGACCCGAG 22	54.5
T.aust_5p_20162		SUT_hvu_mir_000043 1 GGCGGATGTAGCCAAGT-TGAG 21 .||||.|...||.|.|| |..| T.aust_5p_20162 1 AGCGGCTTGCGCGACGTGTTGG 22	54.5
T.tepi_5p_29936		SUT_hvu_mir_000043 1 GGCGGATGTAGCCAAGTTGAG- 21 .||||..||.||...|..||. T.tepi_5p_29936 1 CGCGGCGGTGGCGTTGCCGAAC 22	50.0
T.aust_3p_64344		SUT_hvu_mir_000043 1 GGCGGATGTAGCCAAGTT--GAG 21 ...| ||.|.|||||.. |.| T.aust_3p_64344 1 CATG-ATATCGCCAAGGGACGCG 22	52.2
T.tepi_5p_2711		SUT_hvu_mir_000043 1 GGCGGATGTAGCCAA--GTTGAG 21 . |||||.||.|.|. ||.|.| T.tepi_5p_2711 1 C-CGGATATAACTATCCGTCGGG 22	56.5
T.tepi_5p_13698		SUT_hvu_mir_000043 1 GGCGGATGTAGCCAAGTTGAG- 21 .||..|.|..|||.|.||.|| T.tepi_5p_13698 1 AGCAAAAGCTGCCTAATTAAGG 22	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.alka_5p_5176	SUT_hvu_mir_000010	SUT_hvu_mir_000010 1 TTTGATTA--ATCCGGTCTCGA 20 ||||.||. ||.|...||..| T.alka_5p_5176 1 TTTGTTTTTGATACAAGCTAAA 22	54.5
T.tepi_5p_21088		SUT_hvu_mir_000010 1 TTTGATTA---ATCCGGTCTCGA 20 .|||||.. |||.|.|..||| T.tepi_5p_21088 1 ATTGATATCGCATCGGTTACCGA 23	56.5
T.profu_5p_24235		SUT_hvu_mir_000010 1 TTTG--ATTAATCCGGTCTCGA 20 |.|| |..|||||..|..|. T.profu_5p_24235 1 TATGCCAACAATCCGACCGGGT 22	54.5
T.alka_3p_2121		SUT_hvu_mir_000010 1 TTTGATTAATCCGGTC-TCGA 20 .||||| .||\.| ||.. T.alka_3p_2121 1 ATTGATT---GCGGCCATCCG 18	57.1
T.xiam_5p_12633		SUT_hvu_mir_000010 1 TTTGATTAATCCGGTCTCGA 20 .|.||.|. ||\.|.||\. T.xiam_5p_12633 1 ATGGACTC--CCGCTTTCGC 18	55.0
T.luce_5p_608218		SUT_hvu_mir_000010 1 TTTGATT---AATCCGGTCTCGA 20 |....|| ||.||.| |||| T.luce_5p_608218 1 TGGTCTTTTCAAACCAG-CTCGA 22	56.5
T.xian_3p_9958		SUT_hvu_mir_000010 1 TTTGAT--TAATCCGGTCTCGA 20 ....|| ||..||\.||.|| T.xian_3p_9958 1 AAATATCGTAGGCCGATCACGA 22	54.5

Full sequence-related miRNAs		Sequence alignment	% identity (Stretcher and BLAST)
Bacterial miRNAs	**Barley miRNAs**		
T.luce_3p_384973		SUT_hvu_mir_000010 1 TTT-GATTAAT-CCGGTCTCGA 20 |.| |.||..| |||.|..||| T.luce_3p_384973 1 TGTCGTTTTGTGCCGATAACGA 22	59.1
T.profu_5p_17170		SUT_hvu_mir_000010 1 TT---TGATTAATCCGGTCTCGA 20 || || |.||.|.||....|| T.profu_5p_17170 1 TTGTCTG-TCAAACAGGCAAGGA 22	52.1
T.profu_5p_30378		SUT_hvu_mir_000010 1 TT--TGATTAATCCGGTCTCGA 20 .. |||.|..||||..||.|| T.profu_5p_30378 1 CGGATGAATTTTCCGTCCTGGA 22	54.5
T.luce_3p_186751		SUT_hvu_mir_000010 1 TTTGATTAAT---CCGGTCTCGA 20 .. ||||..| |||.|||.|. T.luce_3p_186751 1 CA-GATTTCTTTGCCGCTCTTGG 22	52.2
T.luce_5p_31255		SUT_hvu_mir_000010 1 TTTGATTAATCCGGTC--TCGA 20 |||..|.|.|...|.| |||| T.luce_5p_31255 1 TTTCCTGACTTTTGGCGGTCGA 22	54.5
T.aust_3p_9648		SUT_hvu_mir_000010 1 TTT--GATTAATCCGGTCTCGA 20 .|| ||||..||.|....||. T.aust_3p_9648 1 CTTTGGATTTGTCGGCAAACGC 22	50.0
T.aust_3p_23871		SUT_hvu_mir_000010 1 TTTGATTAATCCGGTC---TCGA 20 |.||||..|||||..| ||. T.aust_3p_23871 1 TGTGATATATCCGCGCAAATCC- 22	56.5

THE END