**Role of microRNAs in low temperature responses**

by

Swati Megha

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Department of Agricultural, Food and Nutritional Science
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# Abstract

MicroRNAs (miRNAs) are small non-coding RNAs, which are known to regulate plant responses to abiotic stresses, such as Cold Stress (CS) and during normal growth and development. In *Brassica napus* (canola), miRNAs regulate various developmental processes and responses to metal stress however; their role in response to CS is largely unknown. In this study, we investigated CS induced changes in electrolyte leakage, malondialdheyde (MDA), antioxidant enzymes and photosynthetic efficiency in spring canola seedlings exposed to 4°C. Using small RNA sequencing, 70 known and 126 novel miRNAs were identified in CS leaf tissues and, among these, 25 known and 104 novel miRNAs were observed to be differentially expressed. Quantitative real-time (qRT-PCR) analysis of eight selected miRNAs confirmed their CS responsiveness. Furthermore, the expression of six out of eight miRNAs exhibited an opposite trend in a winter variety of canola, 'Mendel', when compared to 'DH12075' which might be a reflection of their cold susceptibility /tolerance.

One of the miRNAs which was observed to be differentially expressed in in *B. napus* in response to CS was miR395. In order to further investigate its role in CS, we cloned the precursor of miR395f from *B. napus*, constitutively overexpressed it in *Arabidopsis thaliana*. Compared with the WT, *A. thaliana* plants overexpressing the precursor of *bna* pre-miR395f displayed a hypersensitive phenotype to freezing stress (-5°C) and CS (4 °C). Increased electrolyte leakage, enhanced MDA content and higher level of staining for reactive oxygen species (ROS) was observed in transgenic lines

indicating altered sensitivity to cold in these pre-miR395f overexpressing lines. Analysis of expression of 18 genes related to sulfur metabolism and those for antioxidant enzymes revealed 14 transcripts to be increased after CS in transgenic lines indicating that both sulfur transport and metabolism as well as the status of sulfur containing antioxidant systems may be altered in the transgenic plants.

In addition, we carried out a study to investigate genetic diversity in 64 accessions of *Brassica* species, including spring *B. napus*, winter *B. napus*, winter *B. rapa* and Recombinant Inbred Lines (RILs) generated from winter × spring *B. napus* crosses using *B. napus* miRNA-SSR markers. In total, 25 miR-SSR markers were mined from 90 known *B. napus* miRNA coding genes. These markers were able to distinguish the *Brassica* lines into five different clusters based on their taxonomic classification and growth habit. All 25 miR-SSRs were found to be polymorphic in the population, however, only the marker miR159-SSR was able to differentiate the winter and the spring growth habit types. These miR-SSR markers exhibited high polymorphism, and grouping of the *Brassica* accessions by cluster analysis was generally consistent with known pedigree suggesting the usefulness of this type of markers for use in breeding and research.

# Preface

A version of Chapter 1 of this dissertation has been published as:

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A version of Chapter 2 of this dissertation has been submitted as a manuscript for publication to Functional and Integrative Genomics as:

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In Chapter 2, 5'RLM-RACE experiment was performed by Dr. Raj Kumar Joshi. For Chapter 4, Dr. Urmila Basu assisted in leaf tissue collection for DNA isolation. In addition, Dr. H. Rahman provided valuable feedback on the data presented in Chapter 4.

I was responsible for conducting all experiments, analysis and interpretation of data, and presentation of the results in the manuscripts of all the studies presented in this dissertation experiments after taking into account feedback from Drs. Kav and Basu. Drs. Kav and Basu (and Dr. Rahman for Chapter 4) reviewed and edited draft versions of these manuscripts.

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Finally, I would like to express my gratitude to my beloved parents and brother Sangam for their constant encouragement and moral support throughout my study.

**To my dearest grandfather!**

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# List of Abbreviations

| | |
|---|---|
| 5'-RLM-RACE | RNA Ligase-Mediated Rapid Amplification of cDNA Ends |
| ABRE | ABA-Response Elements |
| AFLP | Amplified Fragment Length Polymorphism |
| CaMV | Cauliflower Mosaic Virus |
| CAT | Catalase |
| CBF | C-repeat Binding Factor |
| cDNA | Complementary DNA |
| Chl-a | Chlorophyll a |
| Chl-b | Chlorophyll b |
| *COR* | Cold-responsive |
| CPM | Counts per million |
| CS | Cold Stress |
| CSD | Ctosolic superoxide dismutase |
| CSP | Cold Shock Protein |
| DAB | 3, 3'-Diaminobenzidine |
| DCL1 | Dicer Like-1 |
| EL | Electrolyte leakage |
| FW | Fresh Weight |
| GO | Gene Ontology |

## List of Abbreviations (cont.)

| | |
|---|---|
| GPX | Guaiacol Peroxidase |
| GSS | Genome Survey Sequence |
| HSP | Heat Shock Proteins |
| *ICE1* | Inducer of CBF Expression 1 |
| LAC | Laccases |
| LT | Low Temperature |
| *LTI* | Low-Temperature Induced |
| LTRE | Low Temperature Responsive Element |
| MDA | Malondialdheyde |
| MFEI | Minimum Free Energy Index |
| miRNAs | MicroRNAs |
| NaCl | Sodium Chloride |
| NAM | No Apical Meristem |
| NBT | Nitroblue Tetrazolium |
| NGS | Next-generation Sequencing |
| nt | Nucleotide |
| PIC | Polymorphism Information Content |
| PIN | PIN-FORMED proteins |
| pre-miRNA | Precursor miRNA |
| QTL | Quantitative Trait Loci |

## List of Abbreviations (cont.)

| | |
|---|---|
| RAPD | Rapid Amplification of Polymorphic DNA |
| RILs | Recombinant Inbred Lines |
| RIN | RNA Integrity Number |
| RISC | RNA Induced Silencing Complex |
| ROS | Reactive Oxygen Species |
| RT-PCR | Real time PCR |
| sRNA | Small RNA |
| SSR | Simple Sequence Repeat |
| TBA | Thiobarbituric Acid |
| TCA | Trichloroacetic Acid |
| TF | Transcription Factor |
| WUE | Water Use Efficiency |

# Chapter 1: Introduction and Literature Review

## Introduction

World population is predicted to increase from the current ~7 billion to ~10 billion people by 2050 (http://www.un.org/). This continued growth in world population requires maintenance of adequate yield from planted crops from already limited resources available for agriculture. Despite advancements in agronomy practices and technology, significant amount of production is lost due to increasingly variable weather patterns associated with climate change (Mickelbart *et al.* 2015). Current climate models predict an increased incidence of extreme temperatures, floods and droughts over the next couple of years accompanied by a decrease in crop yields by around 20% by the year 2050 (http://www.worldbank.org/). Changing climatic conditions stands as major cause of a variety of environmental attacks on the crop plants in the form of various biotic and abiotic stresses.

Abiotic stress conditions such as extreme temperatures (e.g. freezing, cold, heat), drought, flooding, salinity and heavy metals are among the major causes of crop failure worldwide (Budak *et al.* 2015). It has been demonstrated previously that abiotic stresses inhibit seed germination, seedling and root development, photosynthesis, and the resulting oxidative stress further causes reactive oxygen species (ROS) production, thereby damaging the overall plant growth and productivity (Suzuki *et al.* 2014; Zhang, 2015). Low temperature is one such abiotic stress that adversely affects plant growth and

development and plants have evolved various cellular, physiological and molecular mechanisms in response to cold. The mechanism underlying such responses results from the differential production of several transcripts and their associated proteins. Regulation of gene expression at post-transcriptional and post-translational levels plays a pivotal role in mediating plant responses to stress (Budak *et al.* 2015).

MicroRNAs (miRNAs) are one such class of post-transcriptional regulators of gene expression, which have been shown to play a central role in the survival of plants under abiotic stresses. miRNAs are a class of short endogenous non-coding RNAs that base pair with specific targets to either cleave them or repress their translation. A number of studies have identified a large number of gene coding for miRNAs in response to CS, for instance, in *Arabidopsis thaliana* (Liu *et al.* 2008), *Populus* (Zhang *et al.* 2009b; Chen *et al.* 2012), *Oryza sativa* (Lv *et al.* 2010), *Hemerocallis fulva* (An *et al.* 2014), *Solanum lycopersicum* (Cao *et al.* 2014), *Vitis vinifera* (Sun *et al.* 2015) and *Prunus dulcis* (Karimi *et al.* 2016). Moreover, the genes involved in mediating plant responses to stress represent novel targets for development of abiotic stress tolerant crops.

Canola is one of the most important oilseed crops being cultivated worldwide and is the second largest crop grown in Canada with a value of $26.7 billion to Canadian economy in 2017 (http://www.canolacouncil.org/). The killing frosts during seedling development in the spring, and seed maturation in the fall, is a major factor affecting the spring canola production in Canada (McClinichey and Kott, 2008). For example, in May 2011, southern part of the prairies (Manitoba and Saskatchewan) observed cooler temperatures, which resulted in late or no seeding. Subsequent killer frost in September further caused declined

yield (1,600 kg/ha in Manitoba) especially for the late seeded fields as compared to Alberta (2,200 kg/ha) for that year (Canadian Grain Commission, 2011). Thus, there is a need to further understand the precise molecular mechanisms mediating plant responses to stress. Such knowledge may lead to the development of rational strategies aimed at improving cold tolerance of spring canola.

**Research objectives**

The broad objectives of the research study were:

1) To determine the changes in physiological /biochemical parameters as well as in miRNA profile of canola after exposure to CS for different time points (Chapter 2)
2) To functionally characterize a selected miRNA via heterologous expression in *A. thaliana*. (Chapter 3)
3) Development of miRNA-based SSR markers with potential to classify *Brassica* lines with differential responses to CS. (Chapter 4)

## 1.1 Literature Review

The purpose of this article is to review available literature on miRNAs and their role in mediating plant responses to low temperature (LT) stresses. First, we discuss the transcriptional regulation of genes as an adaptive mechanism of plants during LT stress, followed by a section on miRNA biogenesis, their mode of action and involvement in the molecular processes in plants following LT stress. We have also attempted to summarize studies reported in the literature on the generation and characterization of transgenic plants with altered expression of key miRNAs that are known to be involved in mediating tolerance to LT stress in plants. We conclude that additional expression and functional characterization studies will further improve our understanding of the role of miRNAs in the adaptive mechanisms of plants to LT stresses. This enhanced knowledge could be very useful in the design of rational approaches to engineering LT stress tolerance in economically important plants.

Abiotic stresses such as drought, salinity and temperature extremes adversely affect growth and productivity of agricultural crops. Cold is among the major abiotic stresses, which significantly reduces yield and affects almost every aspect of the physiology and biochemistry of plants (Josine *et al.* 2011; Sanghera *et al.* 2011). Low temperature (LT), including chilling (0-10°C) and freezing (< 0°C) is known to impact the survival and geographical distribution of plants (Josine *et al.* 2011). Although temperate plants do not display freezing tolerance they are known to be chilling tolerant (Josine *et al.* 2011). Exposures to chilling temperatures increase their freezing tolerance by a process known as 'cold acclimation' (Levitt, 1980; Thomashow, 1999). Contrary to this, plants from

tropical/sub-tropical regions such as rice, maize, corn, cotton, tomato are chilling sensitive and do not have the capacity to cold acclimatize (Thomashow, 1999). Moreover, cold acclimation is associated with modifications in plant cell membranes, increased levels of Reactive Oxygen Species (ROS) and activation of ROS scavenging systems, proline accumulation, marked changes in gene expression and biochemical pathways affecting photosynthesis (Sanghera *et al.* 2011; Theocharis *et al.* 2012).

Low temperaure imposes stress on a plant in two ways: the effects of LT alone and dehydration of the cells and tissues when cellular water freezes (Beck *et al.* 2007). Specifically, LT affects cell survival, cell division, photosynthetic efficiency, and water transport with subsequent negative impact on plant growth and productivity (Beck *et al.* 2007). As normal cellular functions are disrupted during abiotic stress, a quick and wide reprogramming at the molecular level is required to respond to these disruptions. This reprogramming is the result of transcriptional, post-transcriptional and translational regulation of the expression of stress responsive genes (Jaglo *et al.* 2001; Skinner *et al.* 2005; Van-Buskirk and Thomashow, 2006; Chinnusamy *et al.* 2007; Jeknić *et al.* 2014). Among the key players in the regulation of gene expression in plants are miRNAs, which are abundant, endogenous, small non-coding RNA molecules known to modulate post−transcriptional regulatory processes (Wang *et al.* 2011; Sunkar *et al.* 2012).

### 1.1.1 Cold responsive transcriptional regulation

Over the years, various differential screening and cloning studies (Thomashow, 1999; Jaglo *et al.* 2001) have led to the identification of a number of cold-regulated genes,

including *COR* (cold-responsive), *KIN* (cold-induced), *LTI* (low-temperature induced) or *RD* (responsive to dehydration). Cold-regulated genes constitute about 4% to 20% of the *Arabidopsis* genome (Hannah *et al.* 2005; Lee *et al.* 2005) and include C-Repeat Binding Factors (CBFs), members of the AP2/ERF (APETALA2/Ethylene-Responsive Factor) transcription factor (TF) family, which bind and activate the expression of many *COR* genes (Gilmour *et al.* 1998; Thomashow, 1999). The promoters of *COR* genes have a CRT/DRE (C-repeat/Dehydration Responsive Element) which acts as a binding site for *CBF* proteins (Stockinger *et al.* 1997) (Figure 1.1). The gene products of *COR, KIN, LTI* and *RD* genes may be classified in two distinct categories. The first group includes late embryogenesis abundant proteins (LEA), heat shock proteins (Hsp), antifreeze proteins, lipid transfer proteins, dehydrins and compatible solutes (sugars, free sterols, raffinose, glucosides, proline, glycine betaine) (Szabados and Savoure, 2010; Kaur *et al.* 2011, Megha *et al.* 2014). The second group contains various TFs, which are involved in regulation of signal transduction and expression of cold-inducible genes (Sanghera *et al.* 2011). Many of these proteins and TFs probably play crucial roles in mediating the observed LT stress tolerance of transgenic plants generated in different studies (Sanghera *et al.* 2011). For instance, transgenic plants expressing cold shock protein (CSP), C2H2 zinc finger, Acyl-CoA- binding protein (ACBP), thermal hysteresis proteins/antifreeze proteins and many more showed improved tolerance to LT stress (Vogel *et al.* 2005; Chen *et al.* 2008; Kim *et al.* 2009; Zhu *et al.* 2010). CSPs function as RNA chaperones by destabilizing the secondary structures of RNA (Weber *et al.* 2002). In *A. thaliana*, *AtCSP3* when over-expressed resulted in enhanced freezing tolerance of transgenic plants. The increased freezing tolerance has been attributed to *AtCSP3* acting as RNA chaperon

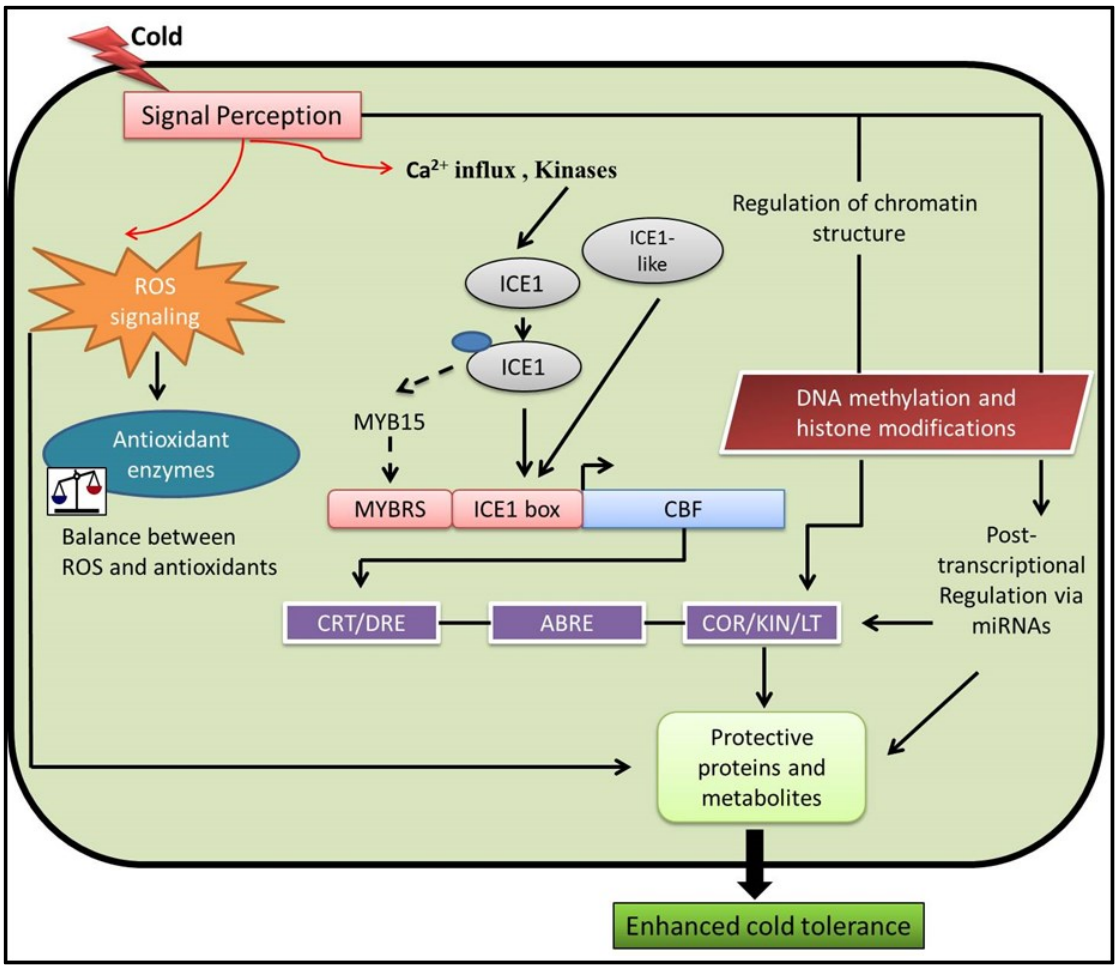


**Figure 1.1: Schematic illustration of regulatory networks involved in low temperature responses.**

Low temperature stress triggers calcium influx and thereby activating protein kinases, which in turn activates *ICE1*. Activated *ICE1* represses *MYB15* and trigger the expression of *CBFs*, which in turn regulates the expression of *COR* genes. The expression of *COR* genes is also regulated by epigenetic changes such as histone modifications and DNA methylation. miRNAs are also involved in regulating the cold stress responsive genes and metabolites at post-transcriptional levels and are also regulated by chromatin changes. Small circles indicate post-transcriptional modification, such as phosphorylation; *ABRE* ABA responsive element, *CBF* C-repeat binding factor, *COR* cold-responsive genes, *CRT* C-repeat elements, *DRE* dehydration-responsive elements, *ICE1* inducer of CBF expression 1, *KIN* cold-induced genes, ROS reactive oxygen species

and thus regulating mRNA stability by mediating RNA duplex formation, which then stabilizes mRNA from exonucleolytic degradation (Kim *et al.* 2009). The over-expression of a Thermal Hysteresis Protein gene, *Thp*1, in *A. thaliana* resulted in plants with low electrolyte leakage and less accumulated Malondialdheyde (MDA), and thus cold-tolerant plants (Zhu *et al.* 2010). Moreover, CS induces *HSP* expression in plants (Timperio *et al.* 2008). These Hsps function in membrane protection, maintaining proteins in their functional conformations, the refolding of denatured proteins and preventing protein aggregation (Timperio *et al.* 2008). Soluble sugars act as compatible solute, by preserving water within the cells, thereby reducing water availability in apoplast for ice nucleation (Ruelland *et al.* 2009). Some pathogen-related (PR) proteins, such as PR1, PR2 (β-1,3-glucanase) and PR5 (thaumatin-like proteins) have been found to have antifreeze properties (Venketesh and Dayanand, 2008). The antifreeze activity of these PR proteins inhibits recrystallization of intercellular ice in the apoplastic space thereby preventing intracellular ice formation (Janska *et al.* 2010). *A. thaliana* Low Temperature-Induced 30 (LTI30) belongs to the group II LEA family and has been shown to be involved in freezing tolerance, possibly by $Ca^{2+}$ signalling (Chung and Parish, 2008). All these studies clearly establish the important role of different cold-regulated genes and their products in modulation of the CS response.

In *A. thaliana*, three *CBF* genes have been identified (Stockinger *et al.* 1997). The *CBF* cold responsive pathway is the best-characterized cold tolerance pathway in plants, with *CBF1*, *CBF2* and *CBF3* (also known as *DREB1b*, *DREB1c* and *DREB1a*) as its main players in *A. thaliana* (Van-Buskirk and Thomashow, 2006; Chinnusamy *et al.* 2007).

Followed by their discovery and functional characterization in *A. thaliana*, CBF homologs have been identified in a variety of monocots and dicots, including rice, wheat, barley, and *B. napus* (Jaglo *et al.* 2001; Choi *et al.* 2002; Dubouzet *et al.* 2003; Vágújfalvi *et al.* 2003; Skinner *et al.* 2005; Jeknić *et al.* 2014). The expression of *CBF* genes is up-regulated in a rapid and transient fashion after cold treatment (Dubouzet *et al.* 2003; Chinnusamy *et al.* 2007; Takuhara *et al.* 2011). Studies show that the expression of CBFs is regulated by *ICE1, ICE2* (Inducer of CBF expression) and three closely related CAMTA (calmodulin binding transcriptional activators) TFs (Chinnusamy *et al.* 2003; 2007; Fursova *et al.* 2009; Doherty *et al.* 2009; Kim *et al.* 2013). *ICE1* encodes a bHLH (basic helix-loop helix) protein, a constitutive TF, which gets activated at low temperatures and acts upstream of the *CBF3* in cold-responsive pathways (Chinnusamy *et al.* 2003; Zarka *et al.* 2003; Lee *et al.* 2005) (Figure 1.1). Over-expression of *ICE1* and *ICE2* in transgenic plants has been shown to increase the expression of *CBF3* and *CBF2* (Chinnumsamy *et al.* 2003; Fursova *et al.* 2009). CAMTA3 binds to *CBF2* promoter resulting in increased expression of *CBF2* under CS (Doherty *et al.* 2009). *A. thaliana* mutants of CAMTA TF have shown decreased ability to cold acclimate, indicating their role in regulation of *CBF* expression (Doherty *et al.* 2009; Kim *et al.* 2013). It can be concluded from all these studies that although *CBF* genes have similar biological functions, the regulation of their expression is considerably complex.

Over-expression of *CBF* genes enhances the cold tolerance of *B. napus* (Jaglo *et al.* 2001), poplar (Benedict *et al.* 2006), and potato (Pino *et al.* 2007). In *A. thaliana* constitutive over-expression of *CBF1* and *CBF3* has been shown to activate the entire

cascade of known CBF/DREB regulated *COR* genes, even at warm temperatures, and resulted in enhanced freezing tolerance (Jaglo *et al.* 1998; Gilmour *et al.* 2000). Based on results from the transcriptomic and metabolomics studies, it was concluded that the improved stress tolerance of *A. thaliana* plants overexpressing *CBF1* may be due to an accumulation of various beneficial metabolites and through the induction of many stress-responsive genes (Fowler and Thomashow, 2002; Marumyma *et al.* 2004, 2009). However, the constitutive over-expression of *CBF* under the control of the CaMV 35S promoter resulted in a 'stunted' growth phenotype and delayed flowering in *A. thaliana*, *B. napus,* and *O. sativa* (Gilmour *et al.* 2000; Jaglo *et al.* 2001; Ito *et al.* 2006). The use of stress-inducible rd29A promoter instead of the constitutive promoter for over-expression studies with *CBF1*/*DREB1a* minimized the negative effects on plant growth (Kasuga *et al.* 1999; 2004). Interestingly, *CBF* overexpressing plants are also tolerant to salt, drought and heat stress, suggesting that the *CBF* function extends beyond CS tolerance (Kasuga *et al.* 1999; Zhang *et al.* 2009a; Ishizaki *et al.* 2013; Kidokoro *et al.* 2015). In contrast, observations on *A. thaliana* mutants including, *eskimo1*, which display enhanced freezing tolerance without prior cold treatment, have suggested the existence of *CBF*-independent cold acclimation pathways. Such mutants exhibited no changes in expression of *CBF* components, but showed a high level of proline accumulation (Fowler and Thomashow, 2002). Epigenetic regulation is also an important mechanism that is involved in an array of biological phenomenon such as genome stability, chromatin regulation, and developmental programming (Feng *et al.* 2010). Chromatin regulation mediated by histone modifications and DNA methylation, is involved in maintaining gene and genome activities (Kurdistani *et al.* 2004). In *A. thaliana*, knockout of components of histone acetyl transferase (HAT)

complexes showed normal *CBF* expression, but reduced *COR* gene expression, suggesting a role of histone acetylation downstream of the CBFs (Vlachonasios *et al.* 2003). Increase in histone H3 acetylation and nucleosome occupancy at *COR* gene promoters was observed during cold acclimation in *A. thaliana* (Pavangadkar *et al.* 2010). HOS15 in *A. thaliana* encodes a histone deacetylase that controls the expression of *COR* genes. The *hos15* mutant plants have shown to accumulate higher levels of some *COR* genes, but not *CBF* transcripts suggesting that HOS15 acts independently or downstream of *CBF* expression (Zhu *et al.* 2008). These studies suggest that changes in plant metabolism or distinct signaling pathways activate different aspects of cold-responsive gene expression and cold acclimation.

In addition to the reprogramming of gene expression, maintaining metabolic homeostasis through detoxification of ROS is another mechanism that is critical for plant survival under LT stress (Gill and Tuteja, 2010) (Figure 1.1). The detoxifying machinery includes detoxifying proteins such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and guaiacol peroxidase (GPX) as well as the antioxidants glutathione (GSH) and ascorbate (Mittler *et al.* 2004; Gill and Tuteja, 2010; Choudhury *et al.* 2016). Readers are referred to excellent reviews for a detailed understanding of role of ROS machinery in LT stress tolerance (Gill and Tuteja, 2010; Choudhury *et al.* 2016).

### 1.1.2 MicroRNAs: discovery, biogenesis and mechanisms

**MiRNA Discovery**

The first miRNA (lin-4) was discovered in the nematode *Caenorhabditis elegans* and was considered as small temporal RNAs (stRNAs) at that time (Lee *et al*. 1993). In the year 2001, because of their observed regulatory roles, these stRNAs were given a formal name, miRNAs, and were classified as a separate distinct class of RNAs (Lagos-Quintana *et al.* 2001; Lau *et al.* 2001; Lee *et al.* 2001). Efforts of four groups in mid-2002 reported the presence of RNAs with miRNA characteristics in *A. thaliana* and thus 19 plant miRNAs (miRNA156 to miRNA 173) were identified 11 years after the discovery of lin-4 in *C. elegans* (Llave *et al.* 2002; Mette *et al.* 2002; Park *et al.* 2002; Reinhart *et al.* 2002). Currently, 8,604 mature miRNAs and 6,882 precursor miRNAs (pre-miRNAs) have been identified in 73 plant species (miRBase, version 21; Kozomara and Griffith-Jones 2014). As alluded to previously, miRNAs are non-coding RNA molecules which are 18-24 nt in length and function as gene regulators in diverse organisms. In plants, these miRNAs affect many biological processes including organ development, phase transition (Chuck *et al.* 2009; Meng *et al.* 2010; Rubio-Somoza and Weigel, 2011; Maizel and Jouannet, 2012; Nova-Franco *et al.* 2015; Kamthan *et al.* 2015; Damodharan *et al.* 2016; Li and Zhang 2016) and in regulating abiotic and biotic stress tolerance (Ni *et al.* 2011; Li *et al.* 2011a; Wang *et al.* 2011; Yang and Chen, 2013; Mondal and Ganie, 2014; Naya *et al.* 2014; Stief *et al.* 2014; Hackenberg *et al.* 2015; Xie *et al.* 2015; Karimi *et al.* 2016; Niu *et al.* 2016). Since the discovery of the first miRNA in 1993, a wide range of studies has provided clear evidence for the involvement of miRNAs in many biological processes including stress responses.

**MiRNA biogenesis**

MiRNAs are transcribed from *MIR* genes, but these transcripts do not get translated to proteins (Coruh *et al.* 2014). The *MIR* loci are independent units and are often located in intergenic regions of genomes (Chen, 2004; Xie *et al.* 2005; Jones-Rhodes *et al.* 2006; Nozawa *et al.* 2012). These loci can be exonic or produced from transposable elements as observed in many plant species including *A. thaliana,* rice and wheat (Piriyapongsa and Jordan, 2008; Li *et al.* 2011b; Lucas and Budak, 2012). Primary transcripts (pri-miRNAs) are generated by the action of RNA polymerase II (Pol II) on *MIR* loci (Bartel, 2004; Xie *et al.* 2010; Kim *et al.* 2011; Bologna and Voinnet, 2014). A 5' 7-methylguanosine cap and a 3' polyadenylated tail are added in order to stabilize the pri-miRNAs (Bartel, 2004; Xie *et al.* 2005; Zhang *et al.* 2005). Reduced pri-miRNA abundance is observed in *A. thaliana* mutants deficient in Cyclin-Dependent Kinase F (CDFK-1). Cyclin-dependent kinase F-1 mediates phosphorylation of largest subunit of RNA polymerase II, which is involved in cap structure in stabilizing pri-miRNAs (Shimotohno *et al.* 2004; Hajheidari *et al.* 2012). pri-miRNA transcripts are cleaved within the nucleus resulting in a characteristic hairpin-like imperfect loop structure called precursor miRNA (pre-miRNA) (Figure 1.2). The pre-miRNA is capping on the nascent transcripts (Shimotohno *et al.* 2004). Thus, impaired CDFK-1 activity reduces mature as well as pre-miRNA abundance, indicating the important role of CDFK-1 in cleaving pre-miRNA to release a miRNA/miRNA* duplex. miRNA* refers to the strand complementary to miRNA, with a 2nt overhang at 3' end of this duplex. Most of the cleavages in miRNA precursors, to form the pre-miRNA and

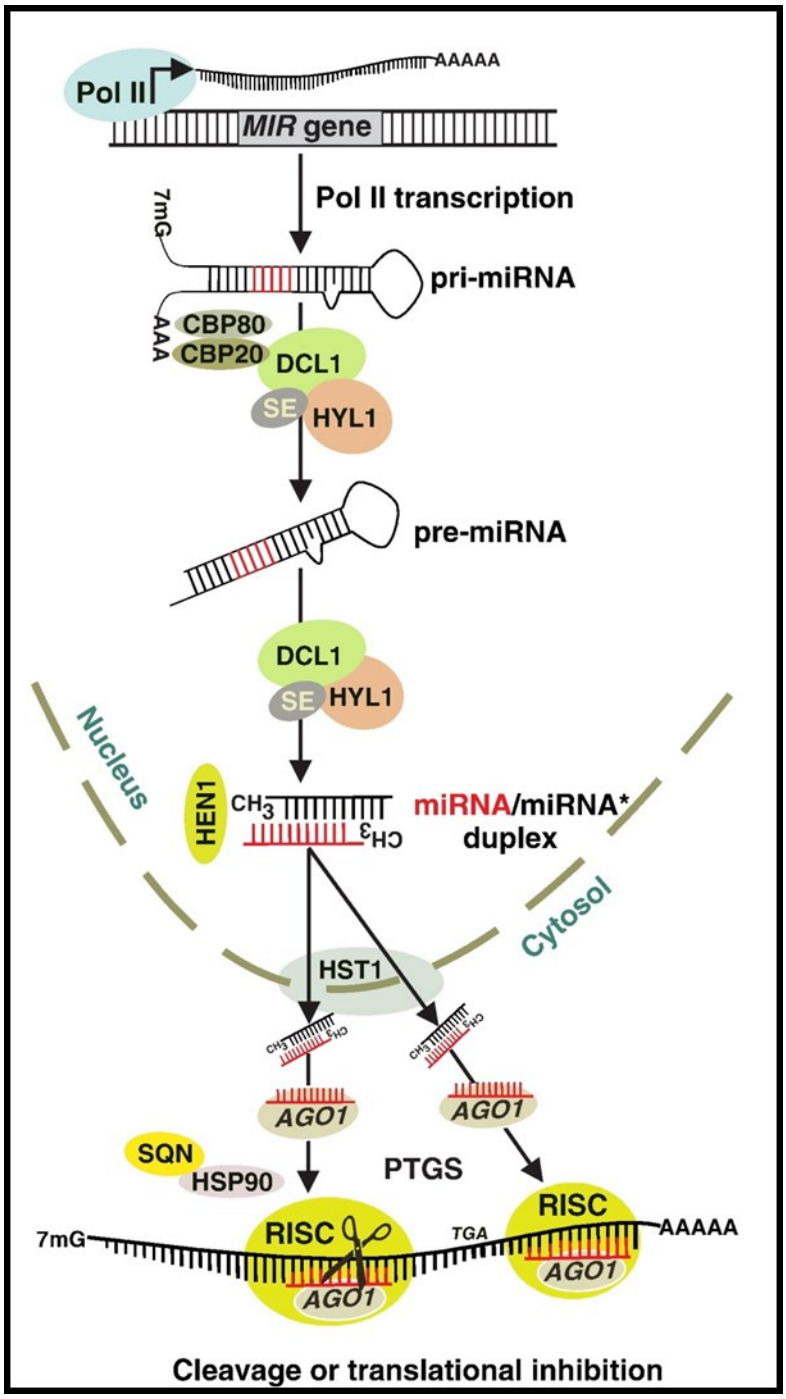


**Figure 1.2: Model for miRNA biogenesis and activity in plants.**

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mature miRNAs, are orchestrated by Dicer Like-1 (DCL1), a type III RNAse which is assisted by the dsRNA binding protein Hypnostic leaves 1 (HYL1) (Han *et al.* 2004; Vazquez *et al.* 2004), zinc finger protein Serrate (SE) (Lobbes *et al.* 2006; Yang *et al.* 2006) and the G-patch domain protein tough (TGH) (Ren *et al.* 2012). Both HYL1 and SE have been shown to improve the efficiency of pri-miRNA processing through *in vitro* biochemical assays (Dong *et al.* 2008). HYL1 binds to miRNA/miRNA* duplex region as a dimer, thereby enabling accurate pri-miRNA processing (Yang *et al.* 2010), whereas zinc finger domain of SE is required for optimal DCL1 activity (Iwata *et al.* 2013). *In vivo* studies show that TGH, a ssRNA binding protein, interacts with both pri- and pre-miRNAs, in addition to its interaction with DCL1, HYL1 and SE, suggesting that it is a crucial component of DCL1 machinery (Ren *et al.* 2012; Ren and Yu, 2012). The 3' end of each strand of miRNA and miRNA* is stabilized by a 2'-O-methylation at the 3'terminal ribose by the nuclear protein HUA1 enhancer (HEN1), thus protecting miRNAs from uridylation and degradation (Boutet *et al.* 2003; Li *et al.* 2005, Yu *et al.* 2005; Zhai *et al.* 2013) (Figure 1.2). Following methylation, the miRNA/miRNA* duplex is exported to the cytoplasm by HASTY, a homolog of animal Exportin 5 (Park *et al.* 2005). In the cytoplasm, one strand of the duplex is incorporated into AGO complex, which then assembles into a functional RNA-induced silencing complex (RISC) driving either mRNA cleavage and/or repression (Mi *et al.* 2008; Montgomery *et al.* 2008). The thermodynamic stability of the 5' end of each strand of duplex determines which specific strand enters the RISC. It has been observed that the strand whose 5' end is less tightly paired is the one that enters the complex, known as guide strand or miRNA, while the miRNA* or passenger strand gets peeled away and is degraded (Khvorova *et al.* 2003; Schwarz *et al.* 2003; Eamens *et al.*

2009; Kwak and Tomari, 2012). The AGO protein contains a PAZ domain (which binds the 3'of guide strand) and a PIWI domain with catalytic residues that confer endonucleolytic activity to the RISC complexes, which are programmed to cleave mRNA transcripts (Baumberger and Baulcombe, 2005; Vaucheret *et al.* 2004, 2006; Iki *et al.* 2010). For a detailed description of miRNA biogenesis in plants, readers are referred to reviews available in the literature (Bartel, 2004; Zhu, 2008; Rogers and Chen, 2013; Ha and Kim, 2014; Bologna and Voinnet, 2014).

**Mechanistic action of miRNAs**

Regulation of mRNA expression by miRNAs happens through two main mechanisms, mRNA cleavage and translational inhibition. The degree of complementarity between miRNA and its binding site within the target decides its mode of action; high complementarity implies miRNA-mediates cleavage of target (Rhoades *et al.* 2002; Mallory *et al.* 2004; Liu *et al.* 2014), while those with low complementarity mediates translational inhibition (Iwakawa and Tomari, 2013, 2015). In plants, the majority of miRNAs have target sites in the open-reading frame (ORFs) and, infrequently, in the 5'-UTRs, 3'- UTRs, or in non-coding RNAs (Addo-Quaye *et al.* 2008; German *et al.* 2008). MiRNAs show extensive complementarity with the target with less than five mismatches and a single G:U wobble. The 5' region from position 2 to 13 is important for plant miRNA-mediated target repression while positions 9 to 11 are critical for AGO slicing (Mallory *et al.* 2004; Schwab *et al.* 2005). Despite the fact that majority of target sites are subjected to AGO1 endonucleolytic cleavage, studies have reported the existence of translational repression in plants (Aukerman *et al.* 2003; Brodersen *et al.* 2008; Lanet *et al.*

2009). It has been observed that, in some instances, translational repression and cleavage pathways may overlap as observed in the case of miR172 family, which regulates the expression of *APETALA2* (AP2) (Aukerman *et al.* 2003). From these studies, it is clear that the regulation of mRNA expression by miRNAs is modulated by different mechanisms, including endonucleolytic cleavage, translational expression or a combination of both.

### 1.1.3 MiRNAs responsive to LT stress

MiRNAs were demonstrated to be involved in the regulation of CS for the first time by Sunkar and Zhu (2004). Small RNA libraries were constructed from *A. thaliana* seedlings exposed to 0°C for 24h and other stresses such as dehydration and salinity. Subsequent RNA gel blot analysis showed strong up-regulation of miR393 expression and down-regulation of miR319c and miR398a expression under CS (Sunkar and Zhu, 2004). Since this initial study, around 17 studies in different plant species have confirmed the role of miRNAs in response to LT stress (Table 1.1). Microarray profiling of miRNAs allowed parallel analysis of a multitude of miRNAs but suffered from a major limitation of its inability to identify novel miRNAs and could not be used for absolute quantification (Pritchard *et al.* 2012). However, microarrays have been successfully used to profile known miRNAs in cold stressed *A. thaliana*, poplar and rice from years 2008-2010 (Table 1.1). Over the years, owing to the technological advancements and availability of genomic sequences for a number of plant species, high throughput, next-generation sequencing methods have become the preferred platform to profile miRNAs under CS (Pritchard *et al.* 2012).

**Table 1.1: List of miRNAs detected and validated through different platforms over the years in different plant species under low temperature stress.**

Asterisk represents non-conserved miRNAs detected in these studies.

| Plant and tissue | **Number of miRNAs up-/down-regulated** | **Number of miRNAs validated** | **References** |
|---|---|---|---|
| *Arabidopsis,* Two week old seedlings 4°C | ↑ 10 / NA | ?? | Liu *et al.* 2008 |
| *Populus tomentosa* (Nisqually-1) 4°C for 24 h | ↑ 15/↓ 4 | *10 | Lu *et al.* 2008 |
| *Arabidopsis,* 3 week old seedlings; 4°C; 0, 1, 2, 6, 12 and 24 h | ↑ 19 / None | 15 | Zhou *et al.* 2008 |
| *Brachypodium distachyon* (ABR5) 12 day old seedlings; 4°C for one week | ↑ 3 , 25* / NA | 3, 8* | Zhang *et al.* 2009b |
| Rice (Prophyll emergence stage) 4°C; 0.5, 1, 3, 6, 9, 12, and 24 h | ↑ 5 /↓ 12 | 5 | Lv *et al.* 2010 |
| *Prunus persica* (Batsch) Non-dormant leaves and chilled dormant leaf buds | ↑ 68 /↓ 10 | NA | Barakat *et al.* 2012 |
| *Populus tomentosa;* 3 months old plants; 4°C for 8 h | ↑ 7, 2*<br>↓ 21 | 19 , 2* | Chen *et al.* 2012 |
| Wheat (BS366); Flag leaf stage | NA | 19 | Tang *et al.* 2012 |
| *Hemerocallis fulva* (Hongbaoshi) 3.5 month old plants; –25 °C for 2 d | ↑ 26 /↓ 30 | None | An *et al.* 2014 |
| *Glycine max* (cv. Williams 82) 4°C for 24 h | ↑ 6 /↓ 5 | 6 | Zhang *et al.* 2014b |
| *Camellia sinensis;* cold tolerant vs. sensitive; 20 day old plants; 4°C; 1, 4, 8, 12, 24, and 48 h | ↑ 31, 46*<br>↓ 43, 45* | 6 | Zhang *et al.* 2014a |
| Tomato (LA1777) 5 leaf stage seedlings; 1, 4, 8, 12, 24, and 48 h | ↑ 12, 11*<br>↓ 20, 6* | 6, 3* | Cao *et al.* 2014 |
| *Prunus dulcis* Mill; Anther and ovary; 0°C for 3h, -1°C for 2 h and -2 for 1 h, consecutively | ↑ 12 /↓ 15 | 16 | Karimi *et al.* 2016 |
| *Citrullus lanatus* L. 4°C for 36 h | ↑ 12 /↓ 20 | None | Li *et al.* 2016 |
| Grapevine (Muscat Hamburg) 6 week plantlets; 4°C; 0 and 4 h | ↑ 7, 4*<br>↓ 29, 4* | 13 | Sun *et al.* 2015 |
| *Glycine max* (Taiwan 75); One-true-leaf stage; 4°C for 24 h | ↑ 21, 30* | 33, 2* | Xu *et al.* 2016 |

Progress on physiological and molecular methods for *de novo* identification of miRNAs in response to abiotic stresses, including cold has been reviewed recently (Begheldo *et al*. 2015). Advances in bioinformatics have made possible the identification and functional annotation of a large number of novel and known miRNAs responding to LT stress from the vast quantities of data generated through RNA-Seq projects (Table 1.1).

Differential profiling of LT-induced miRNAs using microarray and next generation sequencing platforms has been reported from various plant species (summarized in Table 1.1), including *A. thaliana* (Liu *et al.* 2008), *Populus* (Zhang *et al.* 2009b; Chen *et al.* 2012), *O. sativa* (Lv *et al.* 2010), *Hemerocallis fulva* (An *et al.* 2014), *L. esculentum* (Cao *et al.* 2014), *V. vinfera* (Sun *et al.* 2015) and *P. dulci* (Karimi *et al.* 2016). Microarray analysis of LT-treated *A. thaliana* revealed an up-regulation of approximately 8.5% of total miRNAs, with miR408, miR397, miR396, miR393, miR319, miR172, miR171, miR169, miR168 and miR165, exhibiting a fold change of >1.5 (Liu *et al.* 2008). Based on several observations, response of a particular miRNA to the same stress might vary depending on the plant species (Liu *et al.* 2008; Lv *et al.* 2010; An *et al.* 2014; Zhang *et al.* 2014a; Cao *et al.* 2015; Xu *et al.* 2016; Karimi *et al.* 2016). For instance, expression of miR169 was down-regulated in grapevine, rice, wheat, *Populus* (Sun *et al.* 2015; Lv *et al.* 2010; Chen *et al.* 2012; Tang *et al.* 2012), but up-regulated in *A. thaliana, Brachypodium* and almond (Liu *et al.* 2008; Zhou *et al.* 2008; Zhang *et al.* 2009b; Karimi *et al.* 2016) under LT stress. Similarly, LT stress up-regulates miR397 in *A. thaliana, Brachypodium* and *Poncirus* (Liu *et al.* 2008; Zhou *et al.* 2008; Zhang *et al.* 2009a; Zhang *et al.* 2014b), but down-regulates it in grapevine (Karimi *et al.* 2016). MiR398 is down-regulated in grapevine and wheat

(Karimi *et al.* 2016; Wang *et al.* 2014a) but up-regulated in *A. thaliana* and *Poncirus* (Liu *et al.* 2008; Zhou *et al.* 2008; Zhang *et al.* 2014b) in response to LT stress. Moreover, miRNA expression can be also species-specific under LT stress. For instance, in *Brachypodium,* the expression of three conserved miRNAs and 25 *Brachypodium*- specific miRNAs showed significant changes in response to cold stress (Zhang *et al.* 2009b). In another study, 30 cold-responsive miRNAs were identified in *Populus,* of which 27 were conserved and three were *Populus*-specific miRNAs (Chen *et al.* 2012). Quite recently, 17 conserved and 12 grapevine-specific miRNAs were identified after LT stress at 4°C in grapevine (Sun *et al.* 2015).

Different genotypes of one plant species may also vary in their capacity to respond to LT stress and, therefore, the response of miRNAs to LT stress may be genotype specific within the same plant species. Zhang *et al.* (2014a) identified 106 known miRNAs, 98 tea-specific miRNAs and 32 cold-responsive miRNAs through deep sequencing of sRNA libraries from two *Camellia sinensis* cultivars (cold tolerant and sensitive). Of these, 18 and 14 conserved miRNAs were identified from cold-tolerant and sensitive tea cultivar, respectively and included miR171, which is induced in response to LT stress in *A. thaliana* (Liu *et al.* 2008). In this study, expression of miR171 family was up-regulated in cold-tolerant and down-regulated in cold sensitive cultivar; suggesting that miR171 members may perform different functions under LT stress (Zhang *et al.* 2014a). An inverse trend was observed for miR474, which was down-regulated in cold-tolerant and up-regulated in cold-sensitive cultivar (Zhang *et al.* 2014a). In wild tomato cultivar 'LA1777' with high chilling tolerance ability, Cao *et al.* (2014) identified 192 and 205 miRNAs with increased

and decreased expression respectively, after chilling. Despite some variance, similar trends were observed in the expression of six conserved and three novel miRNAs in another chilling tolerant tomato cultivar 'Hezouo908' when subjected to same treatment as LA1777 (Cao *et al.* 2014). Both of these studies suggest that miRNAs may play a cultivar specific role in regulating LT stress tolerance.

Similar to cultivar specific expression of miRNAs, different tissues might show differential expression of miRNAs. For instance, deep sequencing of two sRNA libraries from chilled vegetative buds and young emerging leaves of peach identified 108 miRNAs in both samples, while only 10 miRNAs were specific for buds and 25 miRNAs were unique in leaves (Barakat *et al.* 2012). Chilling stress induced the expression of 17 miRNAs in buds when compared to leaves; with miR167 and miR395 families being the most expressed in buds (Barakat *et al*. 2012). Tissue-dependent expression of miRNAs was also evident under CS in almond, in which miRNA expression profiles were compared between cold-treated anther and ovary samples (Karimi *et al.* 2016). Expression of miRNAs including miR159-5p, miR7723-3p, and miR160f-3p was ovary- as well as cold-stress specific, while miR393 was found to be anther- and cold stress specific. Among differentially expressed miRNAs found in this study (Karimi *et al.* 2016), miR482d-3p showed up-regulation in anther, while its expression was down-regulated in the ovary. In contrast, expression levels of miR172a-5p and miR1511-3p were higher in ovaries and low in anthers; an observation that is corroborated by the fact that miR172 regulates flowering time in *Arabidopsis* (Zhu and Helliwell 2010). Furthermore, the expression of different members of miR156 family (a, b, g, h, i) was down-regulated in both tissue types indicating

the possibility that they may share the same regulatory mechanisms in different tissues (Karimi *et al.* 2016). It can be concluded from these observations that same members of miRNAs may show varied or similar expression patterns in different plant tissues.

In addition to the aforementioned varying expression patterns of miRNAs observed in different tissues, the duration of LT stress may also alter their expression patterns. For example, the expression pattern of miR398 in grapevine and tomato showed a similar downward trend at varying time points (8h, 24h, 48h) under LT of 4°C; but at the 4h time point, expression of miR398 peaked to a 7-fold change only in grapevine (Cao *et al.* 2014; Sun *et al.* 2015). Similarly, when comparing the expression of miR395 in grapevine and *Populus* over a LT stress period of 2-8h, grapevine miR395 showed a slight increase in expression at 2h, while the expression of *Populus* miR395 decreased at 2h (Chen *et al.* 2012; Sun *et al.* 2015). It has also been observed that the expression of species-specific miRNAs can also be affected by the duration of LT stress. For instance, a tomato specific miRNA, miR69.5p, exhibited higher expression after 1 and 8h of stress, whereas it was observed to be down-regulated after 4, 12, 24 and 48h of cold stress (Cao *et al.* 2014). Interestingly, in *Populus,* the expression of cold-responsive pto-miRS16 and pto-miRS16* exhibited inverse patterns, with miRS16* peaking at 8h and miRS16* decreasing at same time point (Chen *et al*. 2012). Differential expression of both miRNA and miRNA* suggests involvement of miRNA* in regulating responses to LT. Other recent findings have found a notably high accumulation of miRNA* and subsequent down-regulation of targets (Okamura *et al.* 2008; Devers *et al.* 2011). These observations suggest that there may be additional factors regulating the expression of miRNAs downstream of their

transcriptional regulation. From these observations, it can be concluded that expression patterns of cold-responsive miRNAs vary with duration of stress as well as the sensitivity/tolerance of a particular plant species towards LT stress.

### 1.2.4 Genes targeted by LT stress responsive miRNAs

MiRNAs do not act directly to modulate plant responses to LT stress. Instead, as stated previously, miRNAs act as regulators of gene expression through endonucleolytic cleavage or translational repression of target genes. Therefore, the identification of target genes involved in LT responses is essential to reveal the regulatory functions of miRNAs as well as to delineate the complex network of genes, which respond to an imposed stress. Both up- and down-regulated cold responsive miRNAs are important in engineering LT stress tolerance in plants, since they may target genes, which may influence cold tolerance in a positive or negative manner. Generally, the up-regulation of a miRNA is associated with decreased expression of its target gene and vice-versa. For instance, under normal growth conditions, miR398 is expressed at optimal levels and, alters the abundance of its target transcripts, Cu/Zn SODs (*CSD1* and *CSD2*) in *A. thaliana* and *O. sativa* (Sunkar *et al.* 2006; Yuzhu *et al.* 2010). Oxidative stress causes down-regulation of miR398 expression both in *A. thaliana*, rice and wheat (Sunkar *et al.* 2006; Yuzhu *et al.* 2010; Wang *et al.* 2014a). And in wheat, accumulation of ROS under LT stress leads to increased levels of ROS detoxifying CSDs, which is further mediated by suppression of miR398 levels (Wang *et al.* 2014a) (Figure 1.3). This inverse relationship between miR398 and its target gene expression has been observed in other cold-stressed plants including tomato (Cao *et al.* 2014) and grapevine (Sun *et al.* 2015). Although no functional studies have

established the direct involvement of miR398 in CS regulation but from the data available, it can be inferred that miR398 regulates expression of CSDs during LT stress. A rice-specific miRNA, miR1425, targets *Rf-1* (Fertility restorer gene), which is a type of PPR (Pentatricopeptide repeat) protein and has been associated with increased cold tolerance of rice at the booting stage (Komori and Imaseki, 2005; Lu *et al.* 2008) (Figure 1.3). *Rf-1* is up-regulated under CS, while miR1425 is down-regulated in rice panicle tissues, suggesting the possible modulation of *Rf-1* expression via miR1425 regulation (Jeong *et al.* 2011). PPR proteins constitute a large family of RNA binding proteins which are known to have a role in processing, splicing, stability, editing and translation of RNA within mitochondria and chloroplasts (Nakamura *et al.* 2012; Manna, 2015). A study in *A. thaliana* has demonstrated that under CS, PPR transcripts were found to have shorter half-lives, which might enable quicker transition of mRNA levels under stress conditions (Chiba *et al.* 2013). Thus, we further suggest that miR1425 regulates cold tolerance by modulating levels of PPR proteins which might help plant to adjust to LT stress, a hypothesis that warrants testing.

The target genes of cold-responsive miRNAs have also been observed to be involved in the regulation of flowering time (e.g. Scarecrow-like protein, Nuclear proteins (Figure 1.4). The differential expression of such miRNA targets also provides additional evidence for crosstalk between gene regulatory pathways involved in plant growth development and those involved in mediating responses to abiotic stress tolerance. All these studies indicate that miRNAs can be potent regulators, which modulate LT responses in different plants by controlling the expression of their target genes.

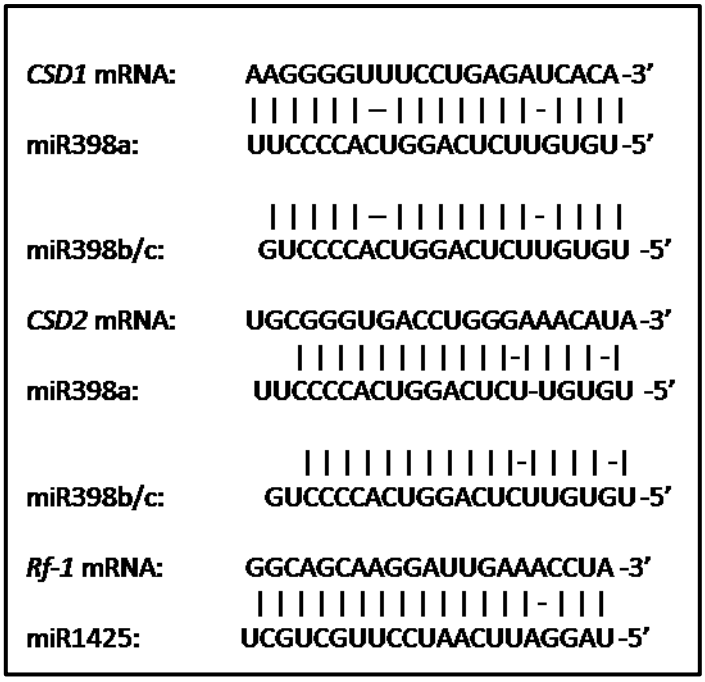


**Figure 1.3: The target site of *A. thaliana* miR398a/b/c and *O. sativa* miR1425.**

The arrows indicate the cleavage sites and localized between the nucleotides 10 and 11 of the miRNA.

### 1.2.5 Case studies: Altering miRNA expression to modulate LT stress tolerance

#### *Role of Arabidopsis miR408 in regulating LT stress tolerance*

MiR408 is a highly conserved miRNA family in land plants with 114 homologues identified in 34 plants till date (Kozomara and Griffiths-Jones, 2014; http://www.mirbase.org/). Differential expression of miR408 in response to various environmental stresses including drought, osmotic and oxidative stress, nitrate, cold, salinity, and mechanical stress, has been well documented (Sunkar and Zhu, 2004;

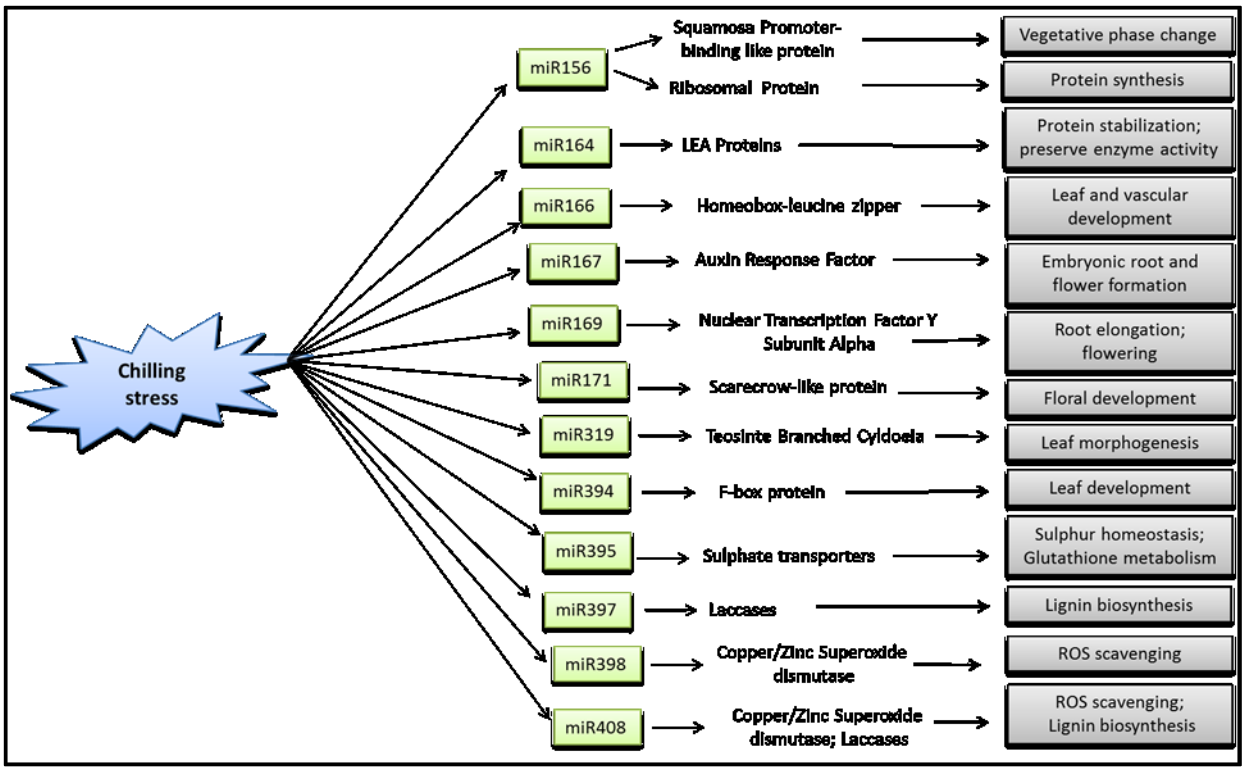


**Figure 1.4: Target genes of miRNAs identified by different groups under CS conditions in various plant species.**

The expression of miRNAs and their targets is up-/down regulated differentially in different crop species, and hence expression pattern is not indicated in the figure.

Trindade *et al.* 2010; Zhou *et al.* 2010; Trevisan *et al.* 2012; Mutum *et al.* 2013; Jovanovic *et al.* 2014; Zhang *et al.* 2014c; Ma *et al.* 2015). Expression of miR408 is also altered in response to different metal stresses including copper, phosphate, calcium, aluminium and manganese (Abdel-Ghany and Pilon, 2008; Valdés-López *et al.* 2010; Lima *et al.* 2011; Mutum *et al.* 2013; Melnikova *et al.* 2014). The *in vivo* targets of miR408 include transcripts for cuproproteins belonging to the phytocyanin family (cupredoxin, plantacyanin and uclacyanin) and laccases *LAC3, LAC12 and LAC13* (Abdel-Ghany and Pilon, 2008).

Members of phytocyanin family contain single copper ion and act as electron transfer shuttles between proteins (De Rienzo *et al.* 2000; Choi and Davidson, 2011). Laccases are glycoproteins containing four copper atoms and catalyze the oxidation of their substrate molecules with the production of water and oligomers, regulating cell wall function (Liang *et al.* 2006). Both phytocyanin family proteins and laccases are primary targets of miR408 and are integral to the regulation of important biological pathways involved in abiotic stress response.

A recent study on miR408 over-expression (OE) in *A. thaliana* reported enhanced LT stress tolerance of *35S:miR408* OE lines (Ma *et al.* 2015). The *35S:miR408* lines exhibited higher survival, low electrolyte leakage, higher $F_v/F_m$ values ($F_v/F_m$ represents the efficiency of photosystem II) and lower levels of MDA, when compared to miR408-KO lines (knockout) and wild type (WT) (Col-0) exposed to -0.5°C in the dark for 12 h prior to being returned to normal growth conditions. In addition, leaf luminescence (a marker for lipid peroxidation levels) and chlorophyll fluorescence were measured to determine cold-induced damage. A lower luminescence and higher chlorophyll fluorescence was observed in miR408-OE plants than in WT and miR408-KO, supporting the idea that elevated levels of miR408 correlates with enhanced LT stress tolerance (Ma *et al.* 2015). This study also measured the expression levels of miR408 and its target genes under CS (-0.5°C for 12 h) in the WT plants. The abundance of *Cupredoxin* and *LAC3* transcripts decreased in accordance with the parallel induction of miR408 expression under CS. It is possible that reduced levels of cuproproteins such as cupredoxin in miR408 over-expression lines might be increasing the endogenous availability of copper for other cuproproteins involved in

mediating responses to abiotic stress, for example, CSDs (Figure 1.5). Consistent with this hypothesis, an increased expression of *CSD1* (cytosolic) and *CSD2* (choloroplastic) was observed in miR408-OE lines (Ma *et al.* 2015). In another related study, a *CBF*-independent nuclear protein, Tolerant to Chilling and Freezing 1 (TCF1) in association with Blue-Copper-Binding Protein (BCB) has been found to regulate lignin biosynthesis in *A. thaliana* (Ji *et al.* 2015). Furthermore, loss of function *TCF1* mutants and *BCB* knockouts had reduced lignin content and increased freezing tolerance. Reduction in lignin deposition in cell walls increases its permeability and also enhances its elasticity allowing it to accommodate growing ice crystals, which may reduce or prevent damage to both the dehydrated cells as well as cell walls (Ji *et al.* 2015). Thus, we hypothesize that a reduced level of *LAC3* transcript would modulate the lignin content by and may be increase the LT tolerance of miR408 overexpressing lines. From all these studies, it is evident that miR408 and the genes involved in copper homeostasis, oxidative stress; lignin biosynthesis and interplay between these molecular processes possibly contribute to LT stress tolerance.

***MiR397a over-expression and LT responses***

In *A. thaliana*, miR397 exists in two isoforms, miR397a and miR397b, both located on chromosome 4 and differing in only one nucleotide (Sunkar and Zhu, 2004). Over-expression of miR397a in *A. thaliana* has permitted the elucidation of its role in regulation of cold signaling pathways and thus tolerance to chilling and freezing stress (Dong and Pei, 2014). Plants overexpressing miR397 continued growing and eventually bolted under a chilling stress of 4°C for two months, when compared to WT plants, which stopped

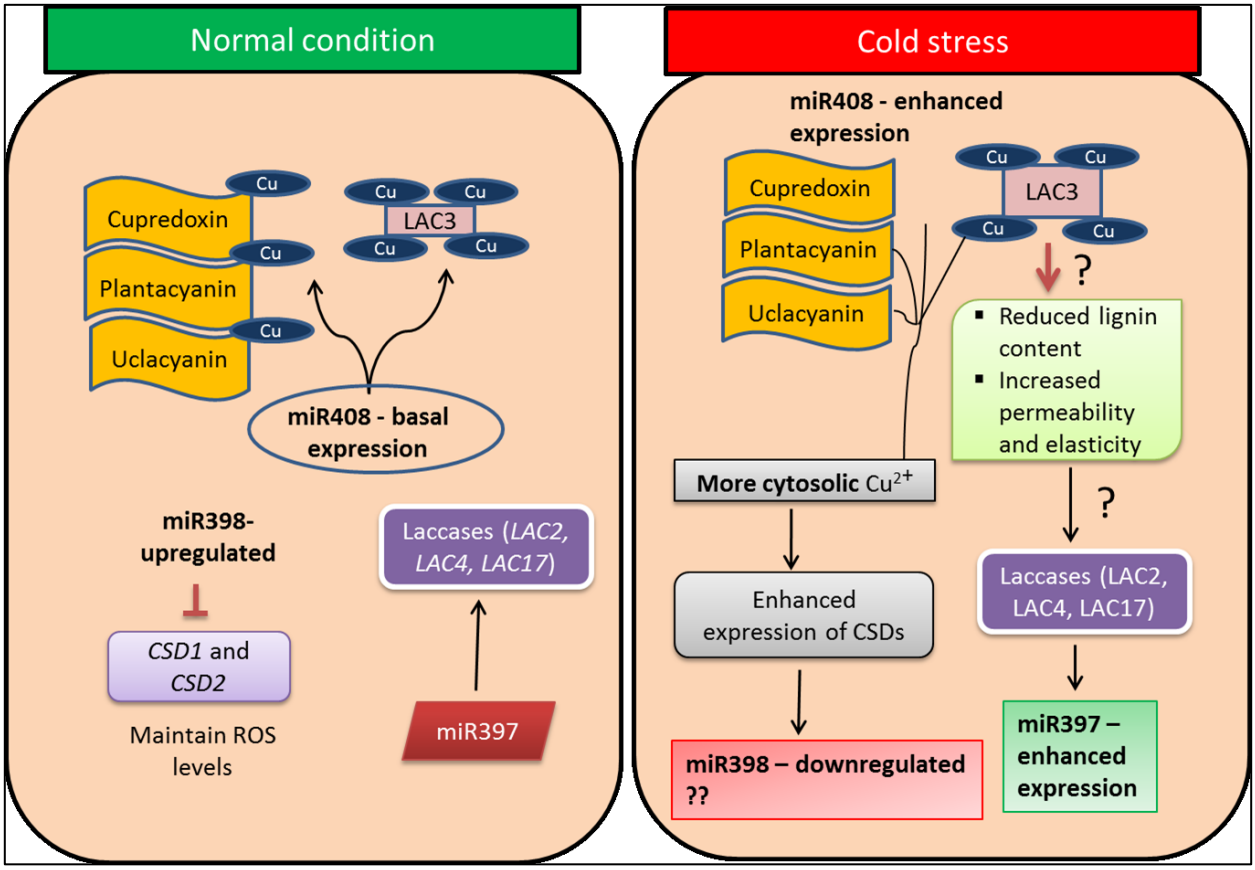


**Figure 1.5: Pictorial representation of genes targeted by miR408 and miR397 under normal growth conditions and when plants are subjected to LT stress.**

Both miRNAs target same members of laccases, and thus it can be hypothesised that these miRNAs increase plant cold tolerance via reduction of lignin content in cell wall, thereby increasing cell wall permeability. Another key player in this mechanism could be miR398, which also targets CSDs. The direct involvement of miR398 in regulation of cold tolerance has not been elucidated yet. CSD cytosolic superoxide dismutase; LAC Laccases; ROS Reactive oxygen species

growing or died under the same stress (Dong and Pei, 2014). Chilling tolerance of miR397a OE lines was further evidenced by a lower leaf electrolyte leakage after 50 days at 4°C. Increased freezing tolerance (-8°C) of OE lines after cold acclimation was based on the survival rate of 90% of miR397a OE plants at -8°C, in contrast to a survival rate of~ 47% for WT plants. Higher transcript levels of cold-induced *CBF* (*CBF1, CBF2 and CBF3*) and downstream cold responsive genes in miR397a OE plants alluded to a possible regulatory function for miR397a in the CBF regulon. MiR397 is known to target three laccases (*LAC2, LAC17* and *LAC4*) and a casein kinase β subunit 3 (Sunkar and Zhu, 2004; Li *et al.* 2010). The effect of overexpressing miR397a on subsequent alteration of its target genes is still unknown and need to be investigated. However, as discussed previously, laccases are involved in reducing lignin deposition at cell wall and thereby increasing its permeability and elasticity. In addition to its involvement in lignin biosynthesis, miRNA397a-mediated laccase expression might play other important roles in plant development and regulation of abiotic stress tolerance. For instance, it has been demonstrated that miR397a increases the number of branches and grain size in rice through the action of a laccase-like gene (Zhang *et al.* 2013). Similar results were also observed in *A. thaliana*, where miR397 OE plants produced enlarged and more seeds (Wang *et al.* 2014b). Furthermore, since both miR408 and miR397 are known to target different members of plant laccases, it would be interesting to investigate further the relationship between these two miRNAs and their targets in mediating plant responses to LT stresses (Figure 1.5).

***Involvement of miR394 in regulating cold stress response in Arabidopsis***

MiR394 is a highly conserved miRNA in both monocots and dicots with 118 homologous members identified till date (Jones-Rhoades and Bartel 2004, Lu *et al.* 2008; Huang *et al.* 2010; Pantaleo *et al.* 2010, Song *et al.* 2012). The *A. thaliana* genome encodes two members of miR394 family (miR394a and miR394b with identical mature sequence) at two genomic loci on chromosome one (Jones-Rhoades and Bartel, 2004). miR394 and its target, *Leaf Curling Responsiveness* (*LCR*), *At1g27340*, a putative F-box protein, have been shown to be involved in the regulation of leaf development, stem cell identity in *A. thaliana* (Song *et al.* 2012; Knauer *et al.* 2013) and fruit and seed development in *Brassica* (Song *et al.* 2015). In addition, miR394 has been implicated in modulating plant responses to salinity and drought stress (Song *et al.* 2013).

Recently, results from an extensive study on over-expression of miR394a and *LCR* in *A. thaliana* have demonstrated the positive role of this miRNA-target pair in response to LT stress (Song *et al.* 2016). Heavy GUS staining was observed in p*miR394a/b::GUS* and *pLCR::GUS* transgenic seedlings treated with cold (4°C) for 12 h, indicating that LT stress induced expression of both miRNA and its target. Interestingly, the *GUS* level was higher than the expression of *LCR* transcripts *pLCR::GUS,* indicating LCR mRNA was being partially silenced by miR394 under CS (Song *et al.* 2016). When subjected to a successive decrease of temperature from 22°C to - 8°C, a cleavage resistant version of LCR mRNA, *35S::mLCR* (with 34.4-40.5 fold increase in the levels of LCR transcript) displayed a lower survival. *LCR* OE lines in *A. thaliana* have shown a decreased expression of auxin flux facilitators, *AtPIN1, AtPIN3, AtPIN4 and AtPIN7* (PIN-FORMED proteins; PIN) (Song *et*

*al.* 2012) and, thus poor survival rate of *lcr* mutant lines could be attributed to this, as CS leads to inhibition of intracellular trafficking of auxin efflux carriers. More specifically, PIN3 efflux carriers are involved in root gravity responses and asymmetric auxin redistribution (Friml *et al.* 2002; Harrison and Masson, 2008) as well as constitutive cycling of PIN2 is involved in the transport of auxin towards the shoots (Paciorek *et al.* 2005, Sukumar *et al.* 2009). We can hypothesize that LT stress causes reduced intracellular cycling of PINs, thereby reducing auxin transport towards shoots and also diminish root's ability to form an auxin gradient (Shibasaki *et al.* 2009). Upon exposure to LT stress (4°C, for 7 days), the *35S:miR394a* OE lines showed 2.0-3.3 fold increase in free proline levels and 1.9-2.1 fold higher total soluble sugars when compared with the WT plants. An increased expression (up to 90 fold) of *CBF3*, in addition to enhanced expression of other cold responsive genes (such as *CBF1, CBF2, RD29A, COR15a* etc.) was also observed in miR394a OE lines (Song *et al.* 2016). *CBF3* OE has been implicated in the alteration of the transcription of Pyrroline-5-Carboxylate Synthase (*P5CS*); thereby increasing free proline content in OE lines (Gilmour *et al.* 2000). Increased free proline and soluble sugar content in both *lcr* mutant lines and miR394 OE lines, when compared to WT plants, suggested independent induction of both miR394a and *LCR* (Figure 1.6). Also, a higher survival rate of 71.7-76.6% was observed in *lcr* mutants, whereas an 88.3-99.3% survival was observed for *35S:miR394a* when compared to WT plants (Song *et al.* 2016). Taken together, these results suggest that both miR394 and its target gene *LCR* are involved in mediating plant responses to LT stress, although the extent of its involvement in CS responsive pathways needs to be investigated further.

***Functional characterization of rice miR319 in LT regulation***

Another key miRNA, implicated to regulate plant responses to various abiotic stresses in various plants including *A. thaliana*, rice and sugarcane, through genome-wide expression analyses, is miR319 (Sunkar and Zhu, 2004; Liu *et al.* 2008; Lv *et al.* 2010; Zhou *et al.* 2010, Thiebaut *et al.* 2012). Detailed investigations into the role of miR319 in regulating LT stress tolerance have been conducted in rice (Yang *et al.* 2013; Wang *et al.* 2014c). The WT plants under LT stress of 12°C or 4°C exhibited a decrease in the abundance of miR319a/b with a corresponding increase in the transcript levels of its targets, suggesting that miR319 might be directly cleaving the targets (Yang *et al.* 2013). Both these studies reported an increase in survival rate of plants over expressing miR319 under CS, when compared to WT plants. Wang *et al.* (2014c) attributed the improved tolerance of miR319 OE (Os-miR319b) plants to enhanced accumulation of free proline, increased expression of LT stress related genes and decreased expression of two target genes; *OsPCF6* and *OsTCP21* (Teosinte Branched Cyldoeia/PCF). In addition, RNAi lines of target genes were generated and they phenocopied the LT tolerance observed in miR319 OE lines as determined by their higher survival rate (Yang *et al.* 2013, Wang *et al.* 2014c), together with increased free proline and ROS scavenging ability (Wang *et al.* 2014c). Similarly, cold inducible expression pattern of miR319 and decreased transcript abundance of *PCF5, PCF6A and GAMyb* was observed in sugarcane (Thiebaut *et al.* 2012). A mechanistic model of regulation of CS tolerance by miR319 and its targets in the *miR319 OE* lines has been proposed (Wang *et al.* 2014c), wherein the over-expression of miR319 under LT stress decreases the transcripts of its targets.

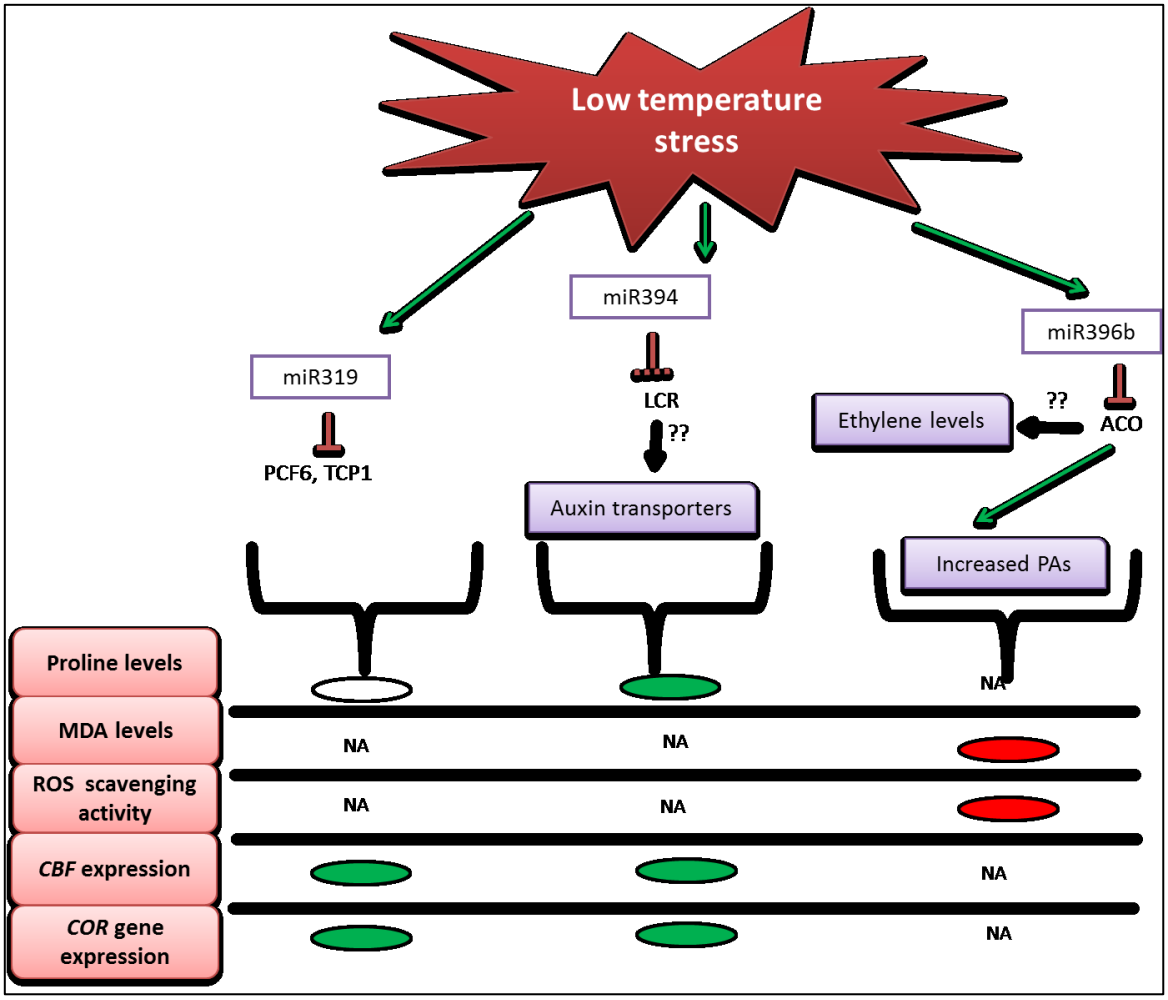


**Figure 1.6**: **Overview of role of three different miRNAs (from over-expression studies) and their respective targets in regulating plant responses to LT stress.**

Low temperature increases the expression of miR319, miR394 and miR396b, which in turn down-regulates the expression of their respective target genes. Increased cold tolerance of plants overexpressing these miRNAs has been marked by increased proline levels, *CBF* and *COR* gene expression and decreased levels of MDA and ROS activity. MDA; Malondialdheyde, ROS; Reactive oxygen species, *CBF*; C-repeat binding proteins, *COR*; Cold responsive, LCR; Leaf Curling Responsiveness; PCF6/TCP1; Teosinte Branched Cyldoeia/PCF, ACO; 1-aminocyclopropane 1-carboxylate oxidase

This leads to the up-regulation of *CBF* genes and ROS-scavenging enzymes and increased cold tolerance (Figure 1.6). Thus, Osa-miR319b, *OsPCF6* and *OsTCP21* can be employed as a potential tool for improving the tolerance of rice to LT stress.

***Role of miR396 in cold tolerance of Poncirus trifoliate (trifoliate orange)***

Trifoliate orange is an extremely cold hardy plant when fully acclimated and ptr-miR396b has been identified as cold-responsive miRNA (Zhang *et al.* 2014b). Over-expression of the precursor of ptr-miR396b in trifoliate orange (Zhang *et al.* 2016) resulted in no noticeable morphological changes with respect to leaf size and shape in miR396b OE plants when compared with WT plants. However, LT stress treatment of OE and WT plants at freezing temperatures (-2°C for 12h) resulted in less serious leaf wilting, significantly lower electrolyte leakage and decreased MDA levels in OE lines, suggesting less severe membrane damage (Zhang *et al.* 2016). To further elucidate the mechanism underlying the enhanced cold tolerance of OE lines, a transient co-expression assay of ptr-miR396b and its target *PtrACO* (1-aminocyclopropane 1-carboxylate (ACC) oxidase; a key gene in ethylene biosynthesis) was performed in *Nicotiana benthamiana* using a green fluorescent protein (GFP)-encoding construct (Zhang *et al.* 2016). No fluorescence was detected in leaf samples co-infiltrated with *35S:miR396b* and *35S:GFP-ACO*, suggesting that *PtrACO* is legitimate target of miR396b and was being cleaved by ptr-miR396b. Moreover, inverse expression patterns of ptr-miR396b (induction) and *PtrACO* (reduction) were observed after LT stress in ptr-miR396b OE lines (Zhang *et al.* 2016). The OE lines also exhibited higher endogenous levels of polyamines and reduced ROS accumulation (Zhang *et al.* 2016) (Figure 1.6). Since ACO is the rate-limiting enzyme involved in

ethylene biosynthesis, a decreased level of ethylene under CS can be based on reduced ACO abundance as observed in this study (Zhang *et al.* 2016). Quite recently, ethylene has been demonstrated as a negative regulatory signal in CS response by targeting C*BF* pathway (Shi *et al.* 2012; Shi *et al.* 2015) and it would be interesting to further elucidate the interplay between ethylene-ACO-miR396b.

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# Chapter 2: Physiological studies and genome-wide microRNA profiling of cold-stressed *Brassica napus*

## 2.1 Introduction

Cold is one of the commonly observed abiotic stresses which affects almost every aspect of the physiology and biochemistry of plants and, as a result, significantly reduces crop productivity (Josine *et al.* 2011, Sanghera *et al.* 2011). Low temperature (LT), including chilling (< 10°C) and frost (< 0°C), imposes stress on plants in two ways: the effects of LT alone, and dehydration of the cells and tissues as a result of the freezing of cellular water (Beck *et al.* 2007). Specifically, low temperature affects cell division, cell survival, and photosynthetic efficiency and thus has a negative impact on plant growth and development (Chinnusamy *et al.* 2010).

As normal cellular functions are disrupted during abiotic stresses, including cold stress (CS), a quick and wide reprogramming at the molecular level is required, which is mediated by transcriptional, post-transcriptional and translational regulation of stress responsive (Chinnusamy *et al.* 2007; Jeknić *et al.* 2014, Megha *et al.* 2017). A series of protective mechanisms against damage by LT stress further induce gene expression changes, including modifications in plant cell membranes, accumulation of antioxidants and cryoprotectants and synthesis of cold-regulated (COR) proteins (Sanghera *et al.* 2011; Theocharis *et al.* 2012, Megha *et al.* 2017). Over the last two decades, numerous cold-induced genes have been identified, functionally characterized and overexpressed to improve plant stress tolerance (Sunkar *et al.* 2012). Owing to the complex and poorly

understood interplay between the genetic pathways, a deeper knowledge is required to understand the possible mechanisms involved in CS tolerance (Theocharis *et al.* 2012).

MicroRNAs (miRNAs) are a class of small non protein coding RNAs, approximately 18-24 nucleotides (nt) long, that function as negative post-transcriptional regulators in eukaryotes (Jones-Rhodes *et al.* 2006; Moran *et al.* 2017). miRNAs are loaded onto the argonaute (AGO) family of proteins to form RNA induced silencing complex (RISC) (Bartel, 2004). Once the RISC complex is formed, the miRNA directs the cleavage or translation repression of target transcript, depending on degree of complementarity between miRNA and its target. If the complementarity is 100%, the miRNA guides the degradation of target and if the complementarity is not 100%, translational silencing of the target gene results (Bartel, 2004). Since the discovery of miRNA in plants, it has been shown that miRNAs regulate the expression of genes/transcription factors induced or expressed during biotic and abiotic stresses as well as in a variety of developmental processes, including auxin signalling, organ morphogenesis and transition from vegetative to floral stage (Sunkar and Zhu, 2004; Khraiwesh *et al.* 2012, Verma *et al.* 2014; Teotia and Tang 2015; Shriram *et al.* 2016; Sattar *et al.* 2016).

Differential profiling of LT induced miRNAs using next generation sequencing and microarray platforms has been reported in various species, such as *Arabidopsis thaliana* (Sunkar and Zhu, 2004), rice (Lv *et al.* 2010), poplar (Chen *et al.* 2012) and soybean (Xu *et al.* 2016). Microarray analysis of LT treated *A. thaliana* revealed an increase in abundance of approximately 17% miRNAs during early stages of cold treatment (Liu *et al.* 2008). In *Populus trichocarpa*, the expression of miR168 and miR477a, b was increased under CS,

whereas miR156g-j, and miR476a were reduced (Lu *et al.* 2008). An attempt was made to understand the role of miRNAs in response to CS, which is one of the major abiotic stresses affecting the rice yields (Lv *et al.* 2010). Eighteen cold-responsive rice miRNAs were identified using microarrays and most of them were found to be down-regulated (Lv *et al.* 2010). Deep sequencing led to the identification of 30 cold-responsive miRNAs in *Populus tomentosa* (Chen *et al.* 2012). Recently, cold responsive miRNAs and their targets were identified in tea (Zhang *et al.* 2014a), soybean (Zhang *et al.* 2014b), tomato (Cao *et al.* 2015), grapevine (Sun *et al.* 2015) and almond (Karimi *et al.* 2016) using high throughput sequencing. Thus, with utilization of microarrays and high throughput sequencing approaches, numerous miRNAs responsive to LT stress have been identified in various plant species.

Canola is one of the most important oilseed crops being cultivated worldwide and is the second largest crop produced in Canada (http://www.canolacouncil.org/). Identification and characterization of novel miRNAs and their targets expressed in response to CS would provide improved understanding of the protective mechanisms working at the molecular level by which plants can cope with CS. This enhanced knowledge can lead to the development of rational strategies for engineering cold tolerance in commercial canola varieties. To our knowledge, our current study has, for the first time, identified novel/known miRNAs using deep sequencing of small RNA libraries from cold stressed canola tissues. Our results and subsequent analysis will provide novel insights into the regulatory role of miRNAs in response to CS in *B. napus*.

## 2.2 Materials and Methods

### 2.2.1 Plant material and growth conditions

Seeds of spring *B. napus* cv. DH12075 were grown in plastic trays with 32 cell packs containing Metro Mix 290 (Grace Horticultural products, Ajax, ON, Canada) in the growth cabinet with 22°C day/18°C night and 16h/8h day/night photoperiod for three weeks. Three-week-old plants were transferred to a chamber for CS treatment at 4°C under weak light conditions (45–55 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Fully expanded second and third leaves were harvested at 0 h (before exposure to CS) and after1, 2, 4, 8, 24 and 48 h of CS treatment, frozen in liquid nitrogen, and stored at -80°C until further use. Leaves of unstressed plants were also harvested as controls at each time point corresponding to the same developmental stage as the cold stressed plants above. The entire experiment was repeated three times with each replicate consisting of 64 plants, and for each time point, leaves from five individual plants were harvested and pooled.

### 2.2.2 Confirmation of imposition of Cold Stress (CS)

In order to ensure that the CS at 4°C was successful in eliciting changes in gene expression, the expression of two C-repeat Binding factors (*CBF5*, *CBF17*) and two Cold Responsive Genes (*BnCOR25*, *Bn115*) was evaluated using qRT-PCR in both control and cold stressed plants. First strand cDNA was synthesized using Mir-X™ miRNA First-Strand Synthesis Kit (Takara Bio USA, Inc.) in reactions with final volume of 20 µL. For each sample, genes were quantified using three independent biological replicates and duplicates from each biological replicate. Changes in the relative gene expression levels

were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *UBC9* (Ubiquitin-conjugating) gene was used as an endogenous control to normalize the concentration of cDNA used in all qRT-PCR reactions.

### 2.2.3 Measurement of physiological parameters

#### Electrolyte leakage

Electrolyte leakage (EL) from *B. napus* leaves was determined using established protocols (Bajji *et al.* 2002) with a few modifications. Briefly, ten freshly excised leaf discs were rinsed with de-ionized water and incubated in tubes containing 15 mL de-ionized water for 2 h at room temperature (21-24°C). Electrical conductivity (EC) of water (EL1) was measured after 2 h using an electrical conductivity meter (Oakton CON11, Cole Parmer, Canada). Final (EL2) conductivity was obtained after disrupting the cell membranes by heating the samples in a boiling water bath for 30 min and after equilibration at room temperature. Membrane stability was expressed as percent electrolyte leakage = [(EL1/EL2) *100].

#### Lipid peroxidation

In order to evaluate lipid peroxidation of leaves, the thiobarbituric acid (TBA) test to determine malonyldialdehyde (MDA) was used (Murshed *et al.* 2008). Briefly, 0.25 g of frozen tissue was homogenized in 1 ml of 0.1% (w/v) TCA solution and the homogenate was centrifuged at 12,000 g for 15 min. For each sample, 0.5 mL of the supernatant was added to 1 mL 0.5% (w/v) TBA prepared in 20% TCA and the mixture was incubated in a boiling water bath for 30 min. The reaction was stopped by placing the tubes in an ice bath

for 5 min, following which the tubes were briefly vortexed. The amount of MDA-TBA complex (red pigment) was calculated from the absorbance at 532 nm after subtracting the non-specific absorption at 600 nm using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

**Measurement of antioxidant enzyme activities**

To measure the activities of antioxidant enzymes, catalase (CAT) and guaiacol peroxidase (POD), frozen leaf tissue powder (0.2 g) was thoroughly homogenized in 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.8) using a chilled pestle and mortar. The samples were centrifuged at 15,000 g for 20 min at 4°C. The supernatant was kept at 4°C and used immediately to determine enzyme activities and protein content (Murshed *et al.* 2008). Activity of CAT was determined by measuring the depletion of $H_2O_2$ by monitoring a decrease in absorbance at 240 nm. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 15 mM $H_2O_2$ and 20 µL of enzyme extract POD activity was determined by measuring the increase in absorbance at 470 nm for three minutes. The assay mixture contained 100 µL of supernatant, 450 µL of 17 mM $H_2O_2$ and 450 µL of 2% guaiacol.

**Determination of chlorophyll and carotenoids**

Chlorophyll a (Chl-a), chlorophyll b (Chl-b*)* and carotenoid content were analyzed by the modified acetone method (Mittal *et al.* 2012). Frozen tissue (100 mg) was homogenized in 1 mL of 80% acetone and placed at 4°C for 24 h. The crude extract was centrifuged and the supernatant was used to measure absorbance at 663 nm, 645 nm and 470 nm, respectively. Different pigments were estimated using the following formulas as given below:

Chl a = 12.7 ($A_{663}$) – 2.69 ($A_{645}$)

Chl b = 22.9 ($A_{645}$) – 4.68 ($A_{663}$)

Car = $A_{470}$ x 200

**Determination of photosynthetic parameters**

Net photosynthetic rate (µmol $m^{-2}$ $s^{-1}$), transpiration rate (mmol $m^{-2}$ $s^{-1}$), intercellular $CO_2$ concentration (µmol $CO_2$ $mol^{-1}$) and water use efficiency (WUE; %) of second fully expanded leaf of three-week-old seedlings, exposed to cold for 0, 1, 2, 4, 8, 24, and 48 h were monitored using an Infra Red Gas Analyzer (IRGA) (Model LI 6400, LI-COR® Inc, Nebraska, USA). WUE was calculated as the ratio of net photosynthetic rate to transpiration rate (Li *et al.* 2011). Measurements were made on three plants from each of the three biological replicates for each treatment.

**2.2.4 RNA isolation and preparation of small RNA library**

Total RNA was isolated from both control (0 h) and CS leaf samples (1, 2, 4, 8, 24 and 48 h) using TRIzol reagent (Invitrogen, CA, USA) according to manufacturer's instructions. Agilent's 2100 Bioanalyzer Plant RNA Nano chip assay (Agilent Technologies, Santa Clara, CA) was used to determine RNA integrity. Samples with 260/280 nm ratio between 1.8 to 2.0 and RNA integrity number (RIN) greater than 7.0 were used for library preparation. Twenty one small RNA libraries (0, 1, 2, 4, 8, 24 and 48 h x 3 biological replicates) were constructed using Illumina's TruSeq Small RNA preparation kit (Illumina, CA, USA) according to manufacturer's instructions. Briefly, 3' and 5' adaptors

were ligated to 1µg total RNA, followed by RT-PCR and barcoding of each library. All barcoded libraries were pooled and sequenced on Illumina HiSeq-2000 at Genome Quebec, McGill University, Montreal, Canada.

### 2.2.5 Small RNA data analysis

The data generated from Illumina HiSeq-2000 was preprocessed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The sequence reads were assigned to corresponding libraries using specific barcode sequences added to samples during library preparation. Briefly, reads were trimmed for minimum quality, clipped of traces of adapter sequences and filtered for minimum length (17-30 nt) using simple mode of Trimmomatic tool (Bolger *et al.* 2014). The clean sequence reads were then processed using miRDeep2 in order to identify the novel and known miRNAs expressed in *B. napus* (Friedländer *et al.* 2008) along with a composite genome comprised of *B. rapa,* GSS sequences of *B. napus* and *B. oleracea*. miRDeep2 identifies miRNAs based on the miRNA biogenesis model *i.e.* detection of the mature miRNA or any one of its precursor or stem loop sequences (Friedländer *et al.* 2008). In addition, all miRNAs from *B. napus*, the set of known precursor and mature miRNA sequences from above mentioned *Brassica* genomes and all remaining viridiplantae miRNAs from miRBase database (Release 20.0) were also analyzed by miRDeep2. Data normalization was performed using EdgeR (McCarthy *et al.* 2012) following the TMM (trimmed mean of M values) method, which calculates the scale factor using median count after trimming the most extreme count values. We considered miRNAs with a normalized read count (Counts per million, CPM) $\geq 5$, to be differentially expressed (p-value $< 0.01$). Following parameters were taken into account to filter down

miRDeep2 predicted novel miRNAs: 1) detection of star miRNA sequence; 2) pre-miRNA length ≥ 60 nt); 3) Minimum free energy index (MFEI) ≥0.85 using the formula from a previous report (Zhang *et al.* 2008):

AMFE = (MFE / (length of a potential pre-miRNA))*100.

MFEI = ((100 × MFE)/Length of RNA/(G +C))%

MFE of miRNA precursor sequences was calculated using web-based software RNAfold, publicly available at (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The raw RNA-Seq data generated from the small RNA libraries has been deposited at the sequence read archive (SRA) of NCBI (Accession number: SAMN07211283).

### 2.2.6 Identification, enrichment analysis and 5'-RLM-RACE of miRNA target genes

Putative target genes of both novel and conserved miRNAs were predicted by searching *B. napus* unigene DFCI gene index using psRNAtarget with default parameters (http://plantgrn.noble.org/psRNATarget/, Dai and Zho, 2011). BLAST2GO (https://www.blast2go.com/, Conesa and Gotz, 2008) was used to understand the putative roles of predicted miRNA targets. Functional enrichment analysis was performed with GO mapping using molecular function and biological process term databases. To experimentally validate the in silico predicted miRNA–mRNA interactions of psRNAtarget analysis, a modified RNA ligase-mediated rapid amplification of cDNA ends (5'-RLM-RACE) was performed as described by Schwab *et al.* (2005) using Gene Racer Kit (Life Technologies). The amplified product was resolved on 1.2% agarose gel, cloned into the pGEM-T easy vector (Promega, Manheim, Germany) and sequenced to confirm the cleavage site. The primers used for 5'-RLM-RACE are provided in Supplementary File 1.

### 2.2.7 qRT-PCR analysis

The expression pattern of differentially expressed miRNAs, six conserved and two novel miRNAs, was determined using qRT-PCR. Reverse transcription was performed using Mir-X™ miRNA First-Strand Synthesis Kit (Takara Bio USA, Inc.) according to manufacturer's instructions. RT-PCR reactions were set up as described earlier. Mature miRNA sequences were used as forward primer, while universal primer provided with the kit was used as reverse primer in all reactions (Supplementary File 1). The expression data was normalized to *U6* small nuclear RNA expression for miRNAs; while *B. napus UBC9* was used as reference for mRNA targets. All samples were analyzed in duplicate, for all three biological replicates. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to measure the relative expression level across the samples as previously described.

For further comparison, a winter variety of canola, 'Mendel' was subjected to the same CS treatment as DH12075, and qRT-PCR was performed to examine the expression of selected miRNAs and their targets, as described above.

### 2.2.8 Cis-element analysis of miRNA genes

Upstream sequences (1.5 kb) of from the start of annotated precursor-miRNAs were extracted as putative promoter regions and searched in Plant Pan 2.0 database for putative transcription factor binding sites ( http://plantpan2.itps.ncku.edu.tw/) (Chow *et al.* 2015).

## 2.3 Results

To confirm that the plants have indeed experienced CS, changes in the expression of two transcription factors (TFs), *CBF* (*BnCBF5*, *BnCBF*17) and two *COR* genes (*BnCOR25, BnCOR115*) were investigated in both control and cold stressed plants. After one hour of CS, expression of both *CBF* TFs increased dramatically (36-fold for *BnCBF5* and 161-fold for *BnCBF17*), as compared to the control plants. The expression of *CBF* TFs showed a gradual increase up to 4 h (*BnCBF5)*, 8 h (*BnCBF16* and *BnCBF17*); followed by a decrease at 24 h and 48 h (Figure 2.1). In addition, the expression of *COR* genes began to increase after 4 h (for *BnCOR25*) and 8 h (for *Bn115*) of CS (Figure 2.1) indicating that CS treatment was effective. Enhanced expression of *CBF* and *COR* has been reported for *A. thaliana*, canola and barley following low temperature stress (Gao *et al.* 2002; Chinnusamy *et al.* 2007; Jeknić *et al.* 2014).

### 2.3.1 Physiological changes in response to cold stress

#### *Determination of Electrolyte Leakage (EL) and Malondialdheyde (MDA) content*

Cell membrane stability under CS conditions was assessed, by measuring EL and MDA contents from both control and cold-stressed plants (Figure 2.2a). Plants kept at 4°C for 1 h exhibited a significant increase in EL (1.5 fold) when compared with 1 h control plants. A steady increase was observed in the EL from 2 h until 48 h, reaching a maximum of 11.2%.

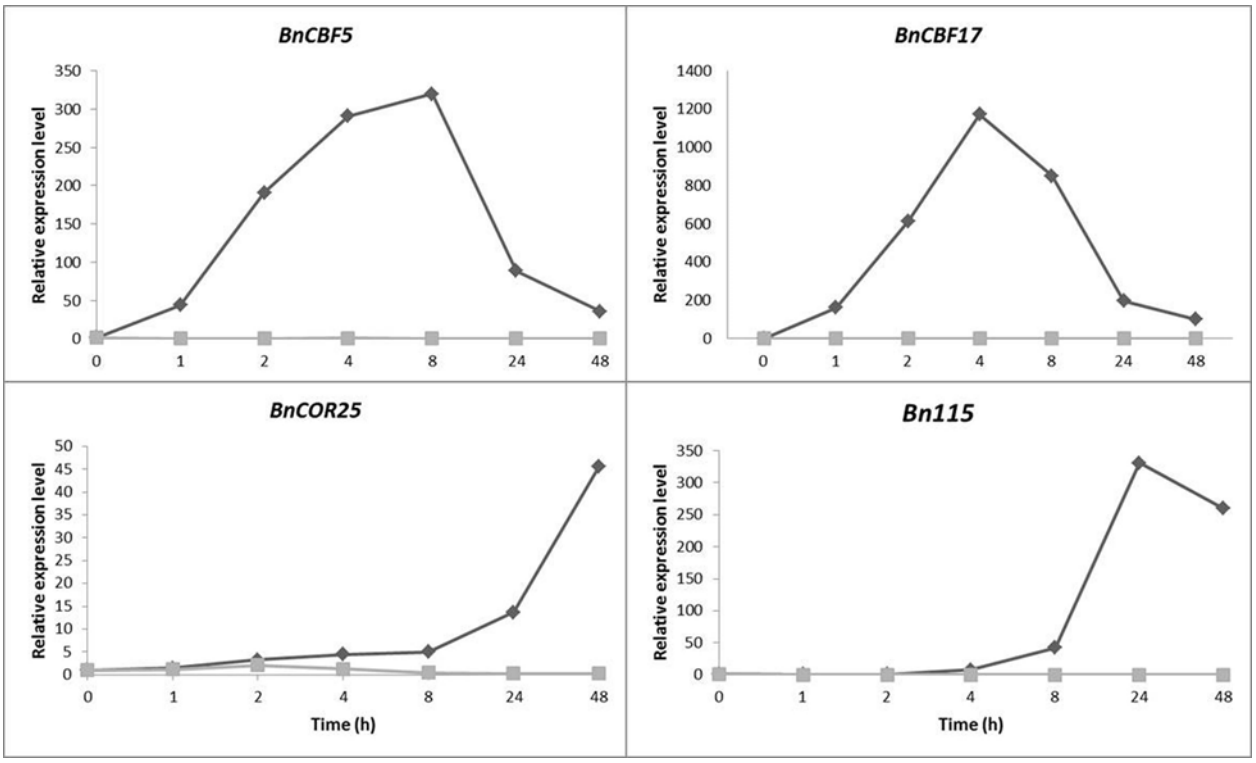


**Figure 2.1: Expression analysis of CBF TFs and COR genes in canola under control conditions and when exposed to 4 °C (CS) for 0-48 h.**

The 'grey' squares represent control growth conditions; while 'black' squares represents CS.

The MDA content of CS plants also showed a significant increase from 1 h to 24 h, peaking at 8 h when compared with control plants (Figure 2.2a). This was followed by a decrease in the MDA content at 48 h after CS. In contrast, control plants exhibited no changes over the course of the 48 h. Our results confirm that plants subjected to CS experienced changes at the physiological level also.

***Determination of antioxidant enzyme activity***

The effect of temperature stress on the levels of antioxidant enzymes was assessed by measuring their activities in both control and plants subjected to CS at 4 °C. Compared with the control, there was a significant increase of 4-6 fold in the levels of CAT enzyme under CS as shown in Figure 2.2a. Furthermore, CAT activity significantly increased steadily with the prolongation of CS until 8 h, followed by a steady decrease over 24 and 48 h. POD activities were also higher in CS plants than controls, except at the 2 h CS time point (Figure 2.2a). POD activity at 1 h CS showed a 2.4-fold increase compared with control at 1 h, followed by a steep decrease at 2 h CS. After 2 h, POD activity in CS plants rapidly increased, eventually peaking at 24 h with subsequent slight decrease at 48 h (Figure 2.2a). This study indicated that both antioxidant enzymes showed elevated levels during the early stages of CS, which declined or remained steady as CS treatment continued.

***Determination of chlorophyll and carotenoid content***

A significant decline in the content of chlorophyll a and b in CS leaves as compared to the leaves of control plants was observed (Figure 2.2a). The range of chlorophyll a and b in control plants conditions was 26.4-27.4 g/g FW, 15.8-23.1 g/g FW, respectively. Under CS, contents of chlorophyll a and b decreased significantly over the duration of stress with a range of 22.9-24.8 µg/g FW and 12.2-14.4 µg/g FW, respectively. Similarly, under CS carotenoid content decreased (7.2-8.3 g/g FW) when compared to control conditions (8.4-10.3 g/g FW).

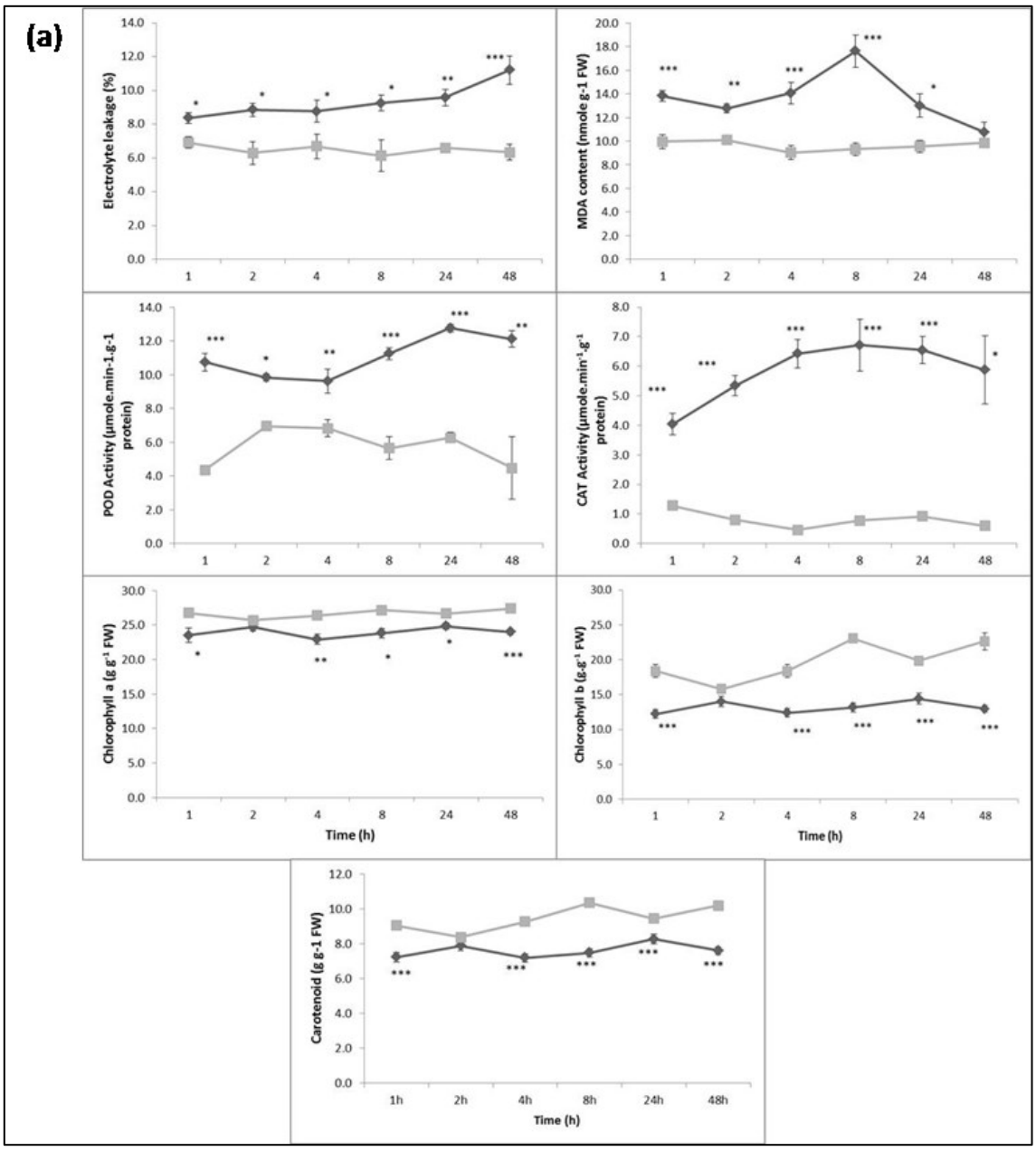


**Figure 2.2a: Changes in electrolyte leakage, malondialdheyde (MDA), catalase (CAT), peroxidase (POD) activity, chlorophyll a, chlorophyll b and carotenoids in response to CS.**

The results are expressed as means with standard error (± SE) of three biological replicates. Statistical significance was determined using a *t* –test. **P*≤0.05>0.005; ***P*≤0.005<0.0001; ****P*<0.0001. FW, fresh weight. The 'grey' squares represent control growth conditions; while 'black' squares represents CS.

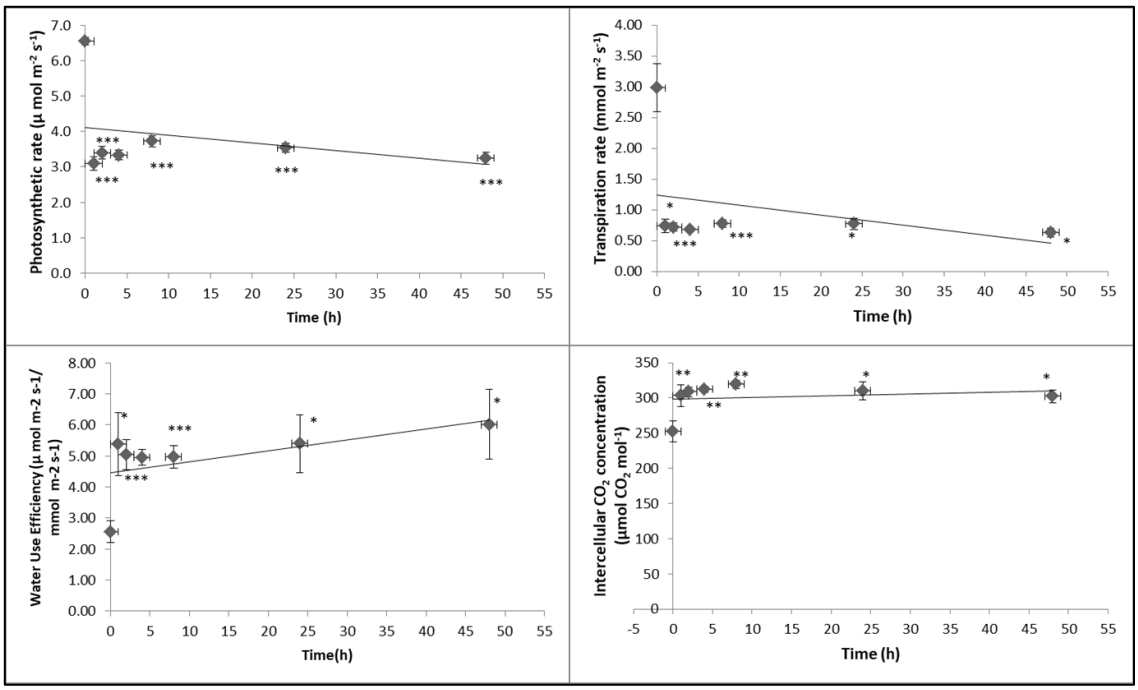


**Figure 2.2b: Photosynthetic indices under cold stress. The results are expressed as means with standard error (± SE) of three biological replicates.**

Statistical significance was determined using a *t* –test. **P*≤0.05>0.005; ***P*≤0.005<0.0001; ****P*<0.0001. FW, fresh weight.

***Determination of photosynthetic activity***

Net photosynthetic rate, transpiration rate, intercellular $CO_2$ concentration and Water Use Effeciency (WUE) of leaves under CS were monitored (Figure 2.2b). Photosynthetic and transpiration rates decreased sharply (~ 50%) after 1 h of CS and stablilized thereafter until 48 h. A similar trend was observed for intercellular $CO_2$ concentration where a significant increase (~ 50%) was observed after 1 h of CS followed by stabilization until 48 h. Further, WUE also peaked at 1 h after CS, following a slight decrease at 2 h, stabilizing at 4 h and 8 h, and finally increasing at 24 h and 48 h (Figure

2.2b). Based on these measurements, it is evident that different parameters of photosynthetic response, increased during the 1 h of CS exposure in *B. napus* plants.

### 2.3.2 Analysis of small RNAs (sRNAs)

Sequencing of sRNA libraries from plants subjected to 0, 1, 2, 4, 8, 24 and 48 h of CS generated 13.4 million to 50.3 million raw reads (Supplementary File 2). Analysis of these reads resulted in identification of 1,953,846 to 4,102,497 clean reads (Supplementary File 2). The remaining reads were either smaller than 17 nt or of low quality (without 3' adaptor sequences), and were excluded from further analysis. Reads with length of 21-24 nt accounted for more than 80% of total number of sRNA reads in all libraries. For libraries from all the time points (Figure 2.3), class of 24 nt sRNAs was the most abundant class (~ 40%), followed by 21 nt (~30%); 23 nt (~8%); 22 nt (~5%). The abundance of 24 nt small RNAs showed a constant pattern in all time points, except after 4 h of CS, where their percentage increased to 50% of the total small RNA population. In contrast, the abundance of 21 nt small RNA population was lowest at 4 h (18 %) (Figure 2.3). Position specific base analysis of identified miRNAs showed presence of Uracil (U) at the first position for miRNAs 21-23 nt, except for 24 nt miRNA in which ~56% miRNAs had Adenine (A) at the first position (Figure S1). Overall, the number of cleaned reads varied in different sRNA libraries, with the highest number of reads of over 21nt sRNAs.

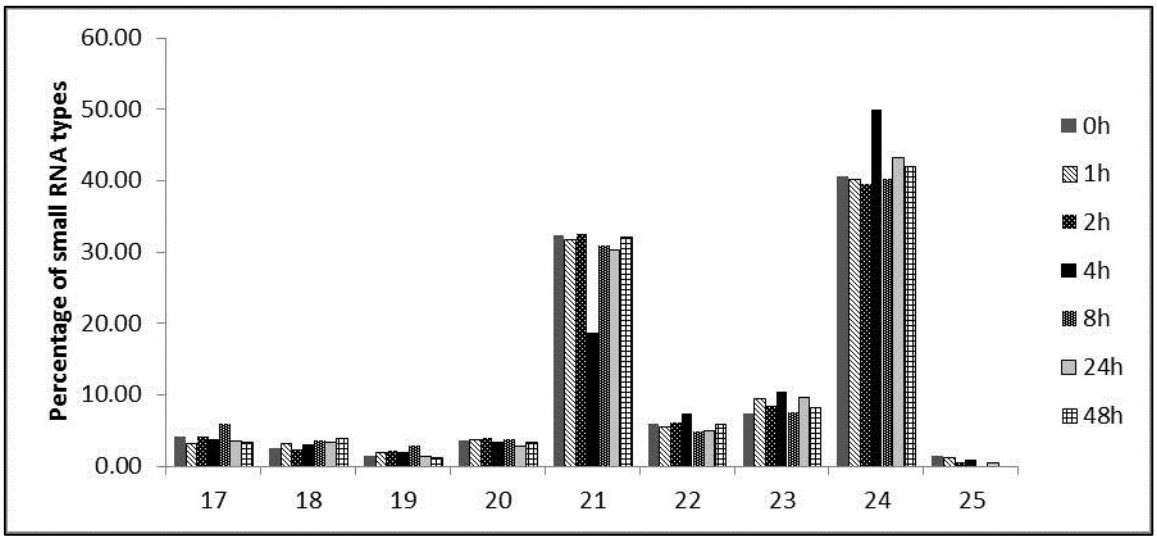


**Figure 2.3: Length distribution of *B. napus* small RNA sequences.**

***Identification of known miRNAs in canola***

To identify the known miRNAs in *B. napus*, all unique sRNA sequences generated from libraries were aligned against miRNA precursors and mature miRNAs in the miRBase (Release 20.0) using BLASTn. All sequences identical to at least one of the previously reported sequences in miRBase were annotated as known miRNAs. A list of CPM and number of members identified for known miRNAs is given in Supplementary File 3. Further, a total of 70 known miRNAs, representing 49 families, were identified from *B. napus* in this study (Supplementary File 3). Conserved miRNAs varied from 20 - 24 nt in length. The average MFE value of the miRNA precursors was 56.20 kcal $mol^{-1}$, and the length of the precursor ranged from 88 - 632 nt, with an average length of 156 nt (Supplementary File 3). In our study, for 11 miRNA families (miR156, miR157, miR159, miR160, miR166, miR168, miR403, miR1140, mir1885b, miR5718, miR5719) more than

ten thousand CPM were detected (Supplementary File 3). The highest read abundance (568,569.30 CPM) was detected for miR166, followed by miR159 with CPM 463,902.9 (Figure 2.4a). For 12 miRNAs, more than one thousand CPM were observed, while 26 miRNAs were detected at a read count of less than thousand. Moreover, the number of members in known miRNA families was also analysed. Out of the aforementioned 43 miRNA families identified, we detected more than one member in 12 families, whereas, only one member was detected in 33 families. The highest number of miRNA members were identified for miR169 (five members) followed by four members each for miR171 and miR395 (Figure 2.4b). This significant distinction in expression abundance of known miRNAs in *B. napus*, as deduced from CPM and number of members identified for individual families, could reflect divergence of miRNA functions during CS treatment.

***Novel miRNAs identified in canola***

A total of 275 sRNAs were predicted to be candidate miRNAs by mirDeep2, based on the alignments of the reads from all seven libraries to a composite genome. Furthermore, presence of corresponding star sequence (miRNA*) and stable hairpin structure of miRNA precursors along with minimum folding free index (MFEI) was used as strict criteria to annotate the identified candidate miRNAs as novel miRNAs. The presence of miRNA* is necessary for the release of miRNA duplex from the predicted hairpin structure (Sunkar and Jagadeeswaran, 2008); thereby supporting the identity of these sRNA sequences as novel miRNAs.

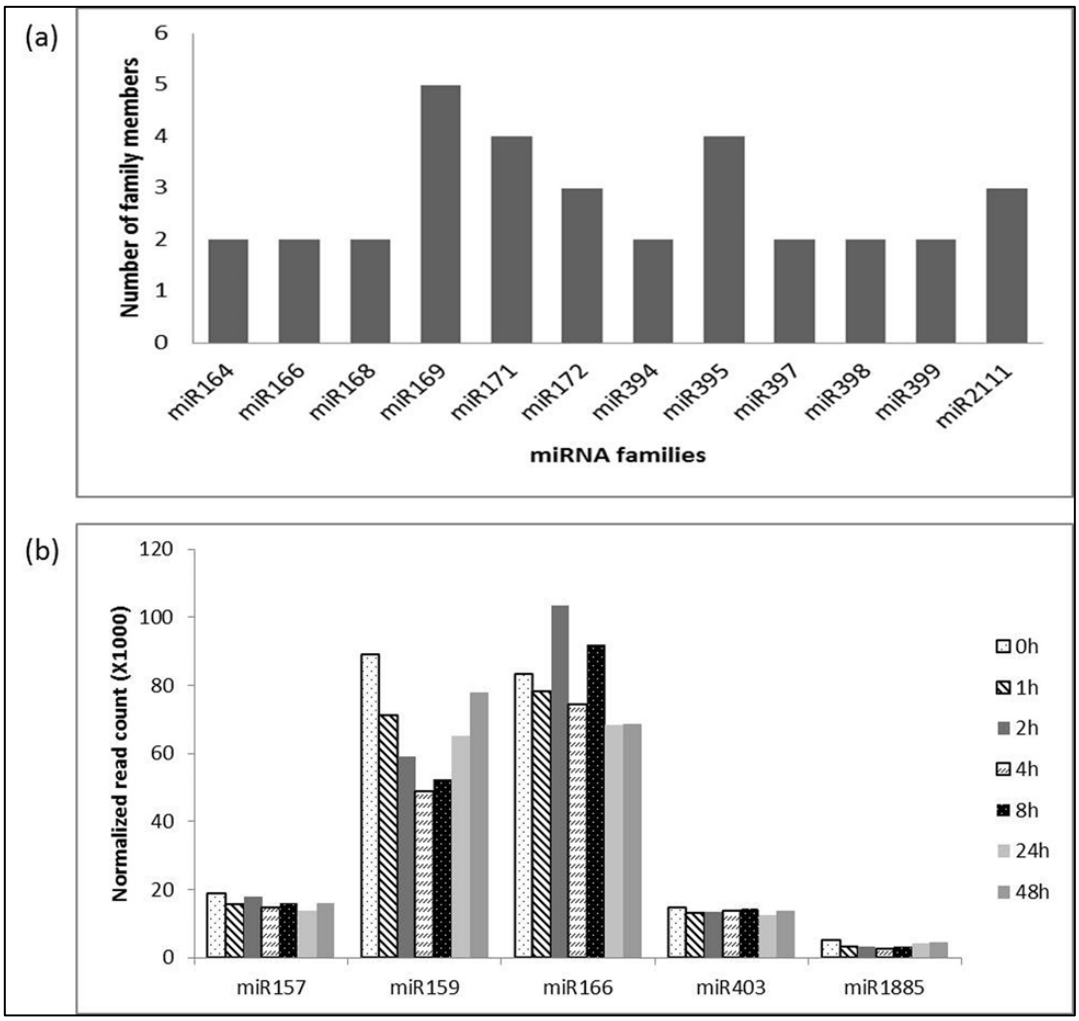


**Figure 2.4: Abundance of known miRNA families and number of members in *B. napus*. (a) miRNA families with more than one member identified in *B. napus*. (b) Normalized read count (CPM) of top five known miRNA families in the small RNA libraries.**

The read count of each family in the control, as well as 1 h, 2 h, 4 h, 8 h, 24 h, and 48 h cold stressed library is shown.

MFEI is an important feature which can be used to distinguish miRNAs from other non-coding RNAs (Zhang *et al.* 2008). MFEI is calculated by the equation: MFEI = (100 × MFE/L)/(G+C) % (L: length of precursor miRNA). In this study, candidate miRNAs with MFEIs ≥ 0.85 were regarded as novel miRNAs and thus 126 sRNA sequences were predicted to be novel based on the above criteria (Supplementary file 4) and have been named as bna-N_miRx (for *B. napus* Novel, x being the number of miRNA). The length of mature, novel miRNAs ranged from 17 - 25 nt, with majority of them being 24 nt long. The length of precursor sequences of novel miRNAs varied from 60 -110 nt, with an average of 80 nt (Supplementary File 4). Furthermore, the MFE of these novel miRNA precursors ranged from -15.2 kcal $mol^{-1}$ to -68.0 kcal $mol^{-1}$ with an average of -33.34 kcal $mol^{-1}$ (Supplementary File 4). The values of MFE for novel miRNAs observed in our study are comparable with the values reported for the precursors of other plant species including *Cicer arietinum* (Kohli *et al.* 2014), *Vitis vinifera* (Sun *et al.* 2015), *Catharanthus roseus* (Shen *et al.* 2017).

Compared with known miRNAs, the CPM of the majority of novel miRNAs was relatively low. However, some novel miRNAs, such as bna-N_miR8, bna-N_miR9, and bna-N_miR22 were detected with one thousand CPM (Supplementary File 4). The CPM for novel miRNAs varied from 3.5 to 25451.3 for bna-novel_miR58 and bna-novel_miR39, respectively. The low abundance of novel miRNAs observed in our study is consistent with earlier notion of the lower expression of novel miRNAs (Zhang *et al.* 2014b, Xu *et al.* 2016).

### 2.3.3 Identification of Differentially Expressed (DE) miRNAs and their target genes under CS

We compared the CPM from cold stressed and control libraries to identify DE miRNAs in response to CS. A total of 25 known miRNAs from 18 families and 104 novel miRNAs were significantly differentially expressed ($p \leq 0.01$; fold change (FC) $\geq$ or $\leq 1$) at different time points. Details of DE miRNAs with fold change and p- values are provided in Supplementary Files 5 and 6. A total of 23 novel miRNAs were DE at all the time points while 82 novel miRNAs were observed to be DE at only one-time point. The majority of conserved miRNAs were down-regulated at different time points whereas, seven miRNAs (miR164d, miR167d, miR168b, miR395c, miR395e, miR398-5p and miR5717) were up-regulated (Supplementary File 6). Twelve novel miRNAs showed a reduced expression at different time points of CS, while rest of the novel miRNAs were up-regulated.

In order to characterize the functions of miRNAs and the downstream effects of transcriptional changes that may be occurring, it is necessary to identify their targets and the specific biological processes or pathways that may be affected by CS. Plant miRNAs have a near-perfect complementarity with their target mRNAs and this criterion is used for miRNA: target predictions (Jones-Rhoades *et al.* 2006; Sunkar and Zhu, 2007). The prediction of miRNA targets for miRNAs in canola was carried out using the web-based program psRNATarget using default parameters. Detailed information on predicted targets of conserved and novel miRNAs is provided in Supplementary Files 7 and 8. A total of 252 putative target genes were identified for 129 DE miRNAs, 67 target genes for 25 conserved miRNAs, 185 target genes for 104 novel miRNAs and no targets for seven novel

miRNAs. Majority of the target genes (73.4%) are predicted to be regulated by transcript cleavage, whereas the remaining targets are potentially regulated by translational repression. Most of the conserved miRNAs had multiple distinct targets, however miR167d and miR394 a, b, appears to target only one mRNA. Out of 104 novel miRNAs, 24 had only one predicted target and the remainder had more than one possible target. The targets of conserved and novel miRNAs included mRNAs encoding auxin signalling F-box 3, ATP sulfurylases, Laccase-like multicopper (LLMO) protein, aminopeptidase, transport inhibitor response (TIR) protein, heat shock protein (HSP), Kelch domain containing F-box protein. In addition, both conserved and novel DE miRNAs were also found to target TFs including *NAC* (for *NAM/ATAF1, 2/CUC2),* Scarecrow-like, and HD-Zip (Supplementary File 7 and 8). Overall, target prediction analysis indicates that the majority of the targets of conserved and novel miRNAs are associated with hormone signalling, developmental processes and stress.

These predicted target genes were annotated with the Blast2GO program and further classified using the WEGO software to better understand their biological functions. Gene Ontology (GO) analysis revealed that they could be classified into 11 cellular components, 24 biological processes and 11 molecular functions. For cellular components, "cell" and "organelle" were the most abundant GO terms. The three most dominant GO terms for biological processes were "cellular process", "metabolic process", and "response to stimulus". With regard to molecular functions, "binding", "catalytic activity" and "transcription regulation" were the three most abundant GO terms (Supplementary Fig S2). GO enrichment analysis further showed that GO terms related to "generation of precursor

metabolites and energy" (GO: 0006091), "response to temperature stimulus (GO: 0009266) and, "response to cold" (GO: 0009409) were significantly enriched (Supplementary file 9). The results from enrichment analysis reveal that predicted target genes may be involved in a broad range of biological processes, in addition to their response to CS.

To validate the computationally predicted miRNA-target interactions in 'DH12075', 5'RLM-RACE was performed for 10 target genes representing five miRNAs (Figure 5) All the predicted targets such as *AFB3, GRR1, TIR1, HSP, APS1, APS4, SULTR2;1* and *F_box* were cleaved at the $10^{th}$ nucleotide position, the same cleavage site frequently observed in plant species (Meyers *et al.* 2008). One of the targets of miR395, *APS3*, was cleaved at $9^{th}$ position, which is similar to previous reports (Verma *et al.* 2014; Kawashima *et al.* 2009). The target of miR397, *LLMO*, showed no cleavage product although an inverse trend was observed for levels of miR397 and *LLMO* by qRT-PCR. This may be due to improper binding of primers used in this study. Overall, 5'RLM-RACE results show that miRNA directed cleavage of targets occurred for selected miRNAs.

### 2.3.4 Expression pattern analysis of DE miRNAs and their predicted target genes

Based on the FC and role of miRNAs and their targets in regulating plant response towards CS, we selected eight miRNAs (six conserved and two novel miRNAs) and their targets to verify their expression patterns in the sequenced spring canola line 'DH12075' (Figure 2.6a). The ability to tolerate CS is a major characteristic which classify canola into winter and spring types. To determine how the expression pattern of these eight miRNAs and their targets differ in winter canola when compared to spring canola, winter canola

variety ‘Mendel’ was used (Figure 2.6b). Expression profile analysis by qRT-PCR confirmed the existence of these miRNAs in both spring and winter *B. napus*, suggesting that these miRNAs may be involved in CS responses. Furthermore, the expression pattern showed opposite trend in ‘DH12075’ and ‘Mendel’ for six out of eight miRNAs tested. For instance, the expression pattern of miR166, miR168, miR394, miR397, bna-N_miR12 and bna-N_miR20 exhibited a down- regulation in cold-stressed ‘DH12075’, whereas these miRNAs were induced in ‘Mendel’ (Figure 2.6). It is tempting to hypothesize that the contrasting trends in miRNA expression patterns between spring line ‘DH12075’ and winter variety ‘Mendel’ might be a reflection of their cold susceptibly/tolerance. To further confirm the variances observed between the spring line ‘DH12075’ and the winter variety, ‘Mendel’, the expression of 12 target genes for eight DE miRNAs was quantified by in ‘Mendel’, with or without CS treatment (Figure 2.7 a and b). The expression of some targets genes such as HD-ZIP III (miR166), RNA recognition motif (RRM) (miR168), Auxin signalling F_box 3 and Transport Inhibitor Response Protein1 (TIR1) (miR393), Laccase like multicopper oxidase (LLMO) (miR397) in CS ‘Mendel’ showed an opposite trend when compared to CS ‘DH12075’. For instance, the expression of Auxin signalling F_box transcript decreased in ‘DH12075’ and increased in ‘Mendel’ with CS. Similar trend was observed for expression of LLMO (target of miR397).

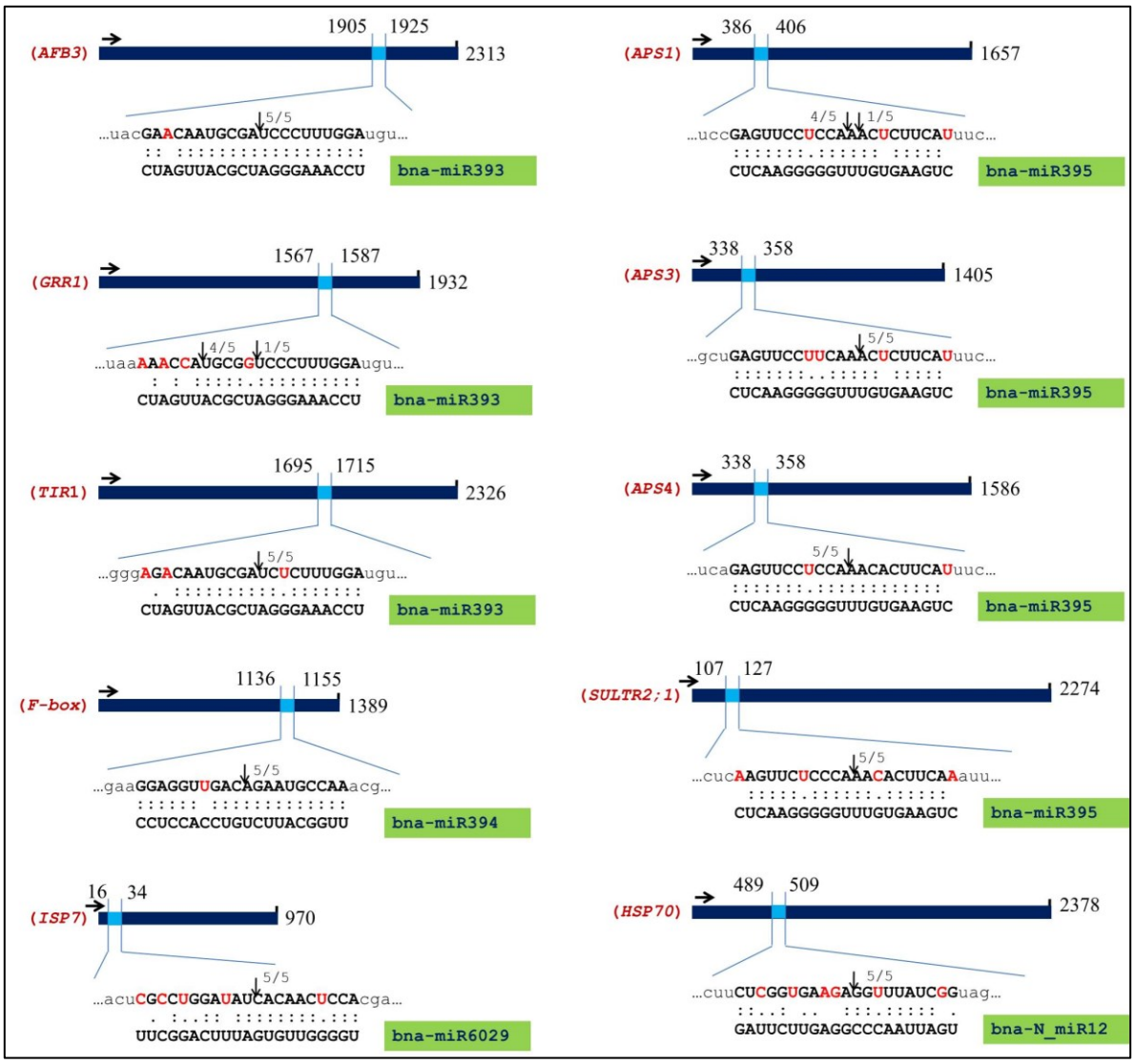


**Figure 2.5: Validation of predicted microRNA (miRNA) targets using RNA ligase-mediated 5′ Rapid Amplification of cDNA Ends (RACE) PCR.**

The authentic targets validated here are from *B. napus*. The bottom strand represents miRNA sequence and the top strand represents a miRNA-complementary site in the target mRNA. Arrow indicates the cleavage position in the target mRNA. Fraction above the arrow refers to the number of independently cloned 5' RACE products whose 5'end terminated at the indicated position over the total number of sequenced clones. Watson-Crick pairing (:) and G:U wobble pairing (.) are indicated. The miRNA and its targets are labeled on the left and right, respectively

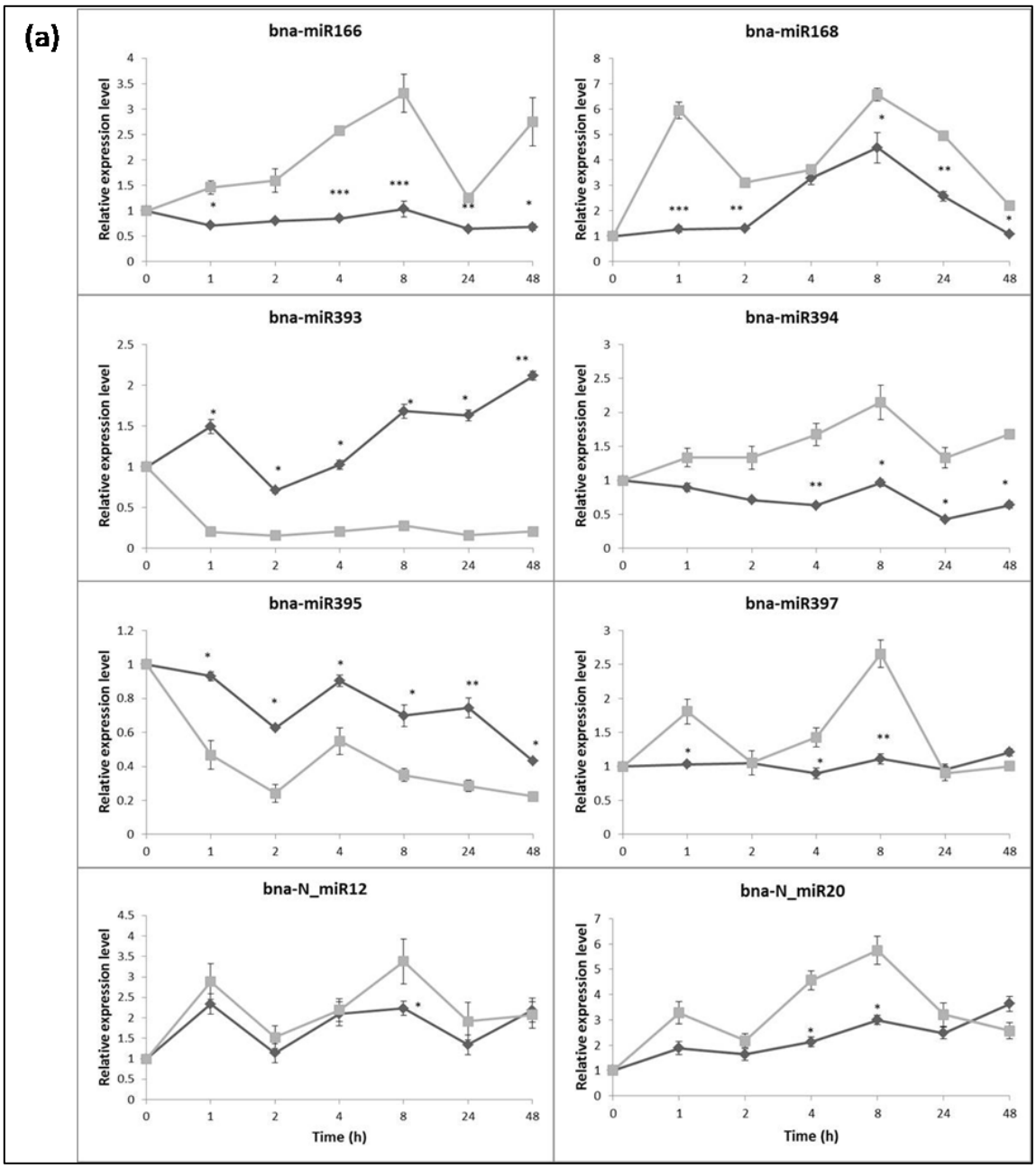


**Figure 2.6a: Expression of chilling responsive conserved and novel miRNAs in 'DH12075' with (CS) or without (control) cold stress treatments.**

The reference gene was *U6*. Normalized miRNA expression amount at 0 h (without cold treatment) was set to 1. Differences between the CS and control time points were tested with a *t* –test. **P*≤ 0.05>0.005; ***P*≤0.005<0.0001; ****P*<0.0001. The 'grey' squares represent control growth conditions; while 'black' squares represents CS.

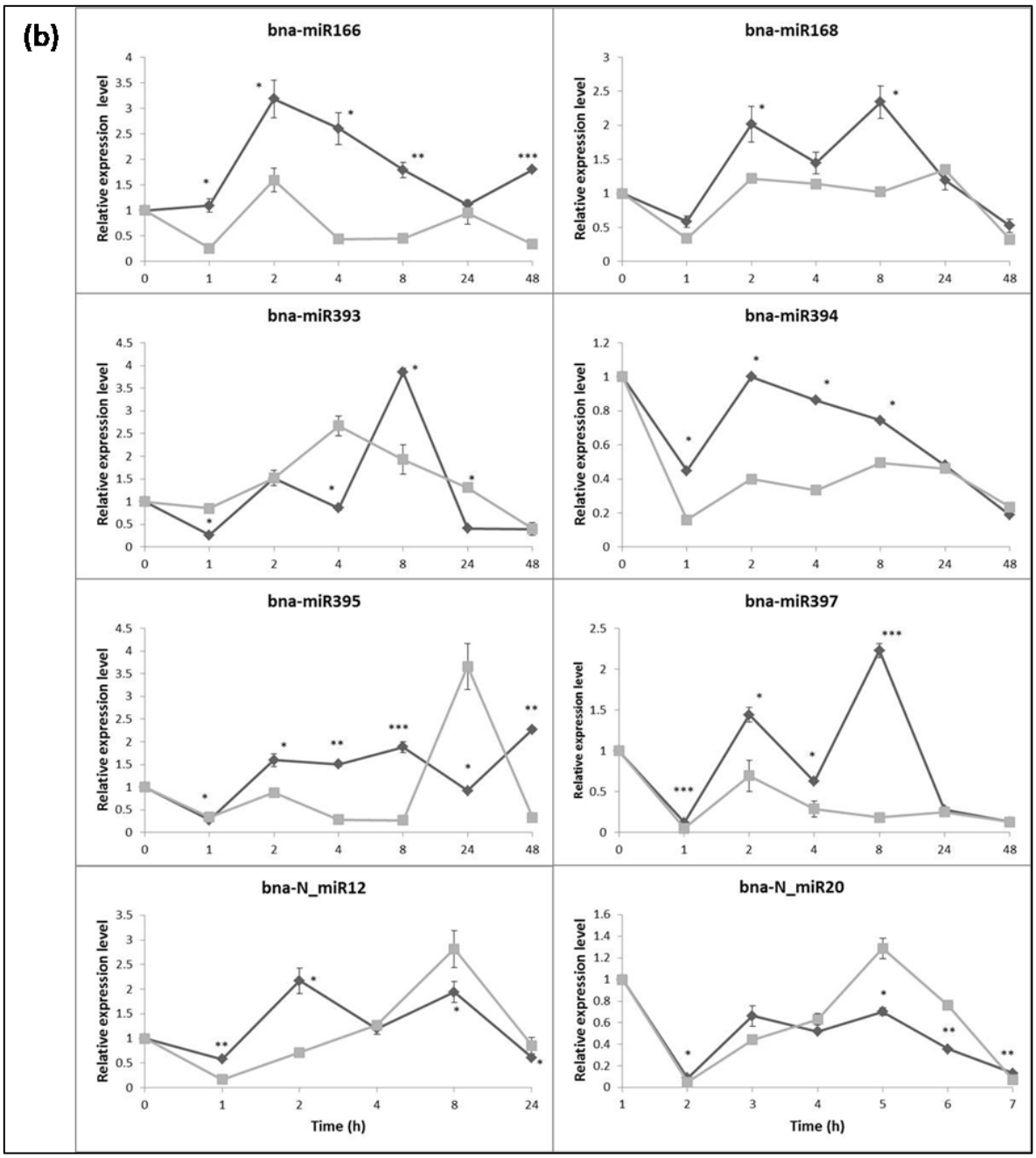


**Figure 2.6b: Expression of chilling responsive conserved and novel miRNAs in 'Mendel' with (CS) or without (control) cold stress treatments**.

The reference gene was *U6*. Normalized miRNA expression amount at 0 h (without cold treatment) was set to 1. Differences between the CS and control time points were tested with a *t* –test. **P*≤ 0.05>0.005; ***P*≤0.005<0.0001; ****P*<0.0001. The 'grey' squares represent control growth conditions; while 'black' squares represents CS.

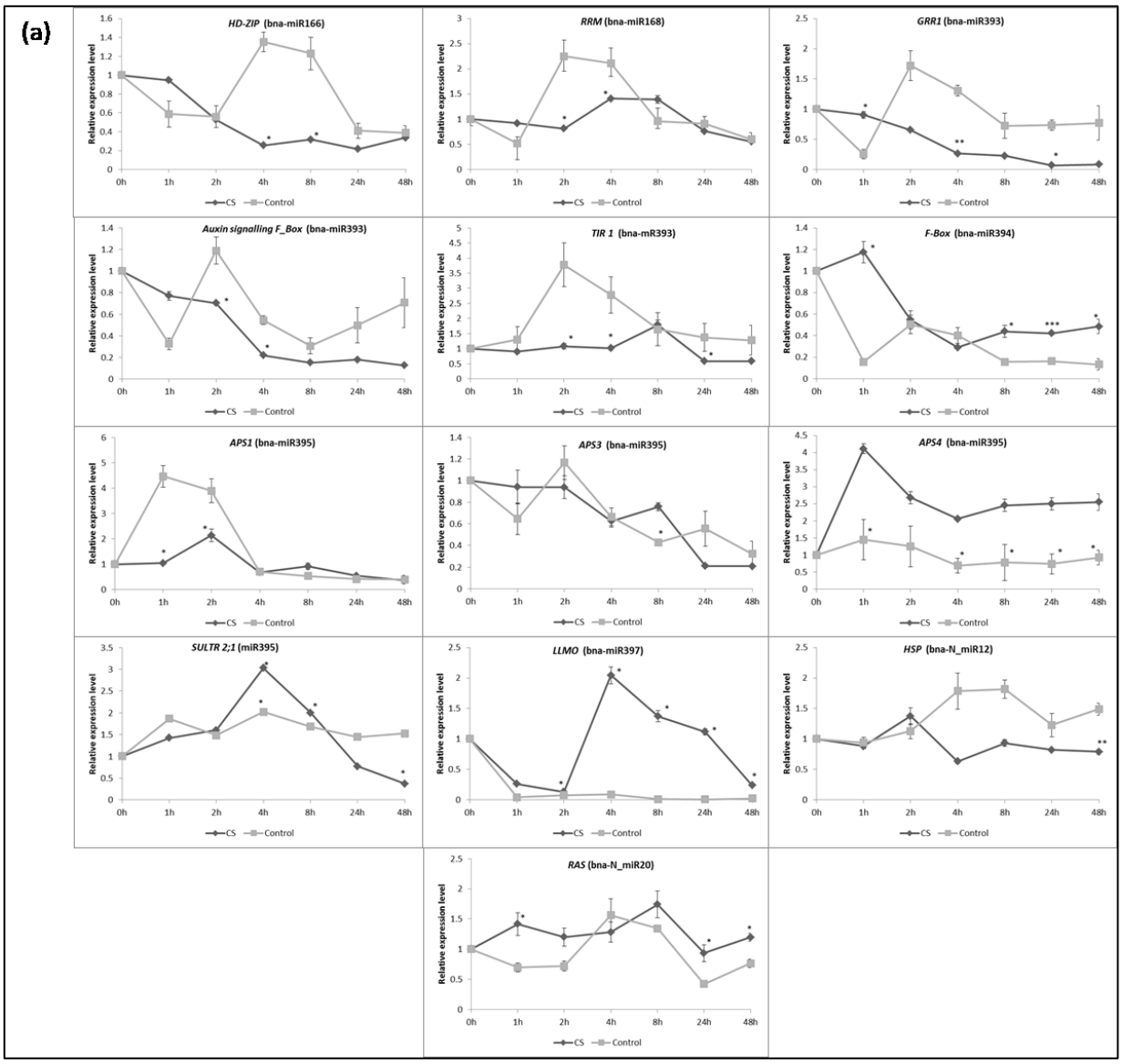


**Figure 2.7a: qRT-PCR validation of miRNA target genes in 'DH12075' with (CS) or without (control) cold stress treatments.**

The reference gene was *UBC9.* Normalized miRNA expression amount at 0 h (without cold treatment) was set to 1. Differences between the CS and control time points were tested with a *t* –test. **P*≤ 0.05>0.005; ***P*≤0.005<0.0001; ****P*<0.0001. The 'grey' squares represent control growth conditions; while 'black' squares represents CS.

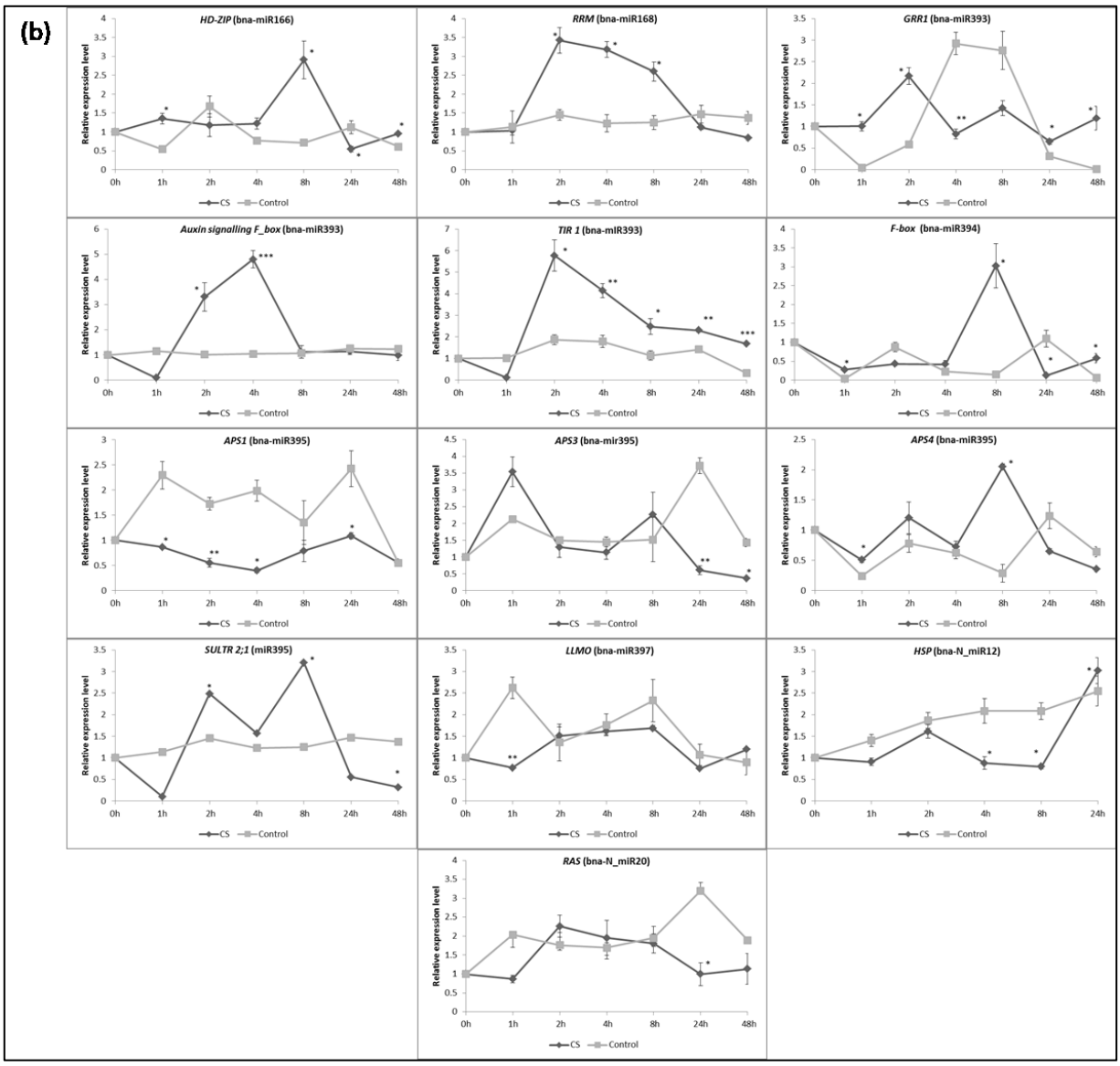


**Figure 2.7b: qRT-PCR validation of miRNA target genes in 'Mendel' with (CS) or without (control) cold stress treatments.**

The reference gene was *UBC9.* Normalized miRNA expression amount at 0 h (without cold treatment) was set to 1. Differences between the CS and control time points were tested with a *t* –test. **P*≤ 0.05>0.005; ***P*≤0.005<0.0001; ****P*<0.0001. The 'grey' squares represent control growth conditions; while 'black' squares represents CS.

### 2.3.5 Analysis of cis-acting elements in the promoters of miRNA genes

To further understand the regulation of DE miRNAs, 1.5kb sequences upstream of 14 known and two novel miRNAs were analyzed for the distribution and occurrence of stress-relevant *cis*-elements. Among the elements listed in Table 2.1, we identified several known stress-responsive elements, including ABA-response elements (ABREs), anaerobic induction elements (AREs), heat stress response elements (HSE), low temperature responsive element (LTRE), binding site for cold-responsive transcription factor (RAV1), binding site of inducer of *CBF* expression 1 (ICE1) (MYC-C) and defense / stress responsive elements (TC-rich repeats). Phytohoromone regulatory elements including gibberellin-responsive elements (GARE); MeJA-responsive motif (CGTCA-motif) and salicylic acid responsive element (TCA-element) were also identified, suggesting that these miRNAs might be regulated by phytohoromones under CS (Table 2.1). The most common motif observed in this study was the MYC-C (present in putative promoter regions of 13 miRNAs with an average abundance of 7.4. This was followed by RAV1 and LTRE, with an average abundance of 6.6 and 4.1 (Table 2.1). In plants, majority of ABA- responsive genes contain ABREs in their promoters (Joshi *et al.* 2017) and in this study ABREATCAL (a $Ca^{2+}$-responsive ABRE) and ABRELATERD1 (Dehydration stress and dark-induced senescence responsive) were detected in seven and ten miRNA genes, respectively. These results indicate that these miRNAs might be involved in responses to other abiotic stresses in addition to CS.

**Table 2.1: Known stress-related cis-elements in the upstream regions of 12 known and 2 novel miRNA genes**

| | ABRELATERD 1 | ABRERATCAL | ARE | CGTCA-motif | GARE | HSE | LTRE | MYC | RAV1 | TCA-element | TC-rich |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **miR168a** | 5 | 3 | 2 | 1 | | 1 | 3 | 11 | 5 | 1 | |
| **miR168b** | 2 | 1 | 1 | 1 | 3 | 2 | | 14 | 4 | 1 | |
| **miR393** | | | | | 4 | 3 | | 2 | 8 | | 1 |
| **miR394a** | 1 | 1 | 3 | | 3 | 2 | | 3 | 4 | | |
| **miR394b** | 3 | 1 | 1 | 1 | 8 | 2 | 3 | 10 | 9 | 2 | 2 |
| **miR395c** | | | 2 | | | 3 | 1 | | 8 | | 2 |
| **miR395e** | 1 | 1 | 2 | 1 | 2 | 1 | 2 | 19 | 6 | 2 | 3 |
| **miR395f** | 2 | | | 1 | | 1 | 21 | 3 | 10 | 1 | 4 |
| **miR397a** | 1 | | | 3 | | | 1 | 11 | 7 | | |
| **miR397b** | 1 | | | 2 | 1 | | 1 | 10 | 6 | | 2 |
| **miR5712** | 1 | 2 | 5 | | 3 | 2 | 4 | 7 | 6 | | 2 |
| **miR6029** | 6 | 4 | 3 | | 1 | | 1 | 3 | 5 | 3 | 2 |
| **bna-N_miR20** | | 0 | 2 | | | 2 | 4 | 1 | 4 | | 1 |
| **bna-N_miR12** | | | 1 | 1 | 1 | 1 | | 2 | 11 | 1 | 2 |
| **Total** | 23 | 13 | 22 | 11 | 26 | 20 | 41 | 96 | 93 | 11 | 21 |
| **Mean in total miRNAs** | 2.3 | 1.3 | 2.2 | 1.4 | 2.9 | 1.8 | 4.1 | 7.4 | 6.6 | 1.6 | 2.1 |

*ABRE-like sequence (from -199 to -195) required for etiolation-induced expression of erd1 (early responsive to dehydration) in *A. thaliana* (**ABRELATERD1**; ACGTG); "ABRE-related sequence" or "Repeated sequence motifs" identified in the upstream regions of 162 $Ca^{(2+)}$ -responsive upregulated genes (**ABRERATCAL**; MACGYGB**);** anaerobic response element (**ARE;** TGGTTT); MeJA-responsiveness element (**CGTCA**-motif); GARE (GA-responsive element); heat stress element (HSE; AGAANNTTCT); low temperature responsive element (LTRE; GTCGG/**CCGAC**); MYC recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in *A. thaliana* (MYC-C; **TAACTG)**; (RAV1; **CANNTG);** SA-responsive element (TCA-element); defense and stress responsiveness (TC-rich repeats).

** The data presented in this table is not enriched for presence of *cis*-elements.

## 2.4 Discussion

Cold stress is a common abiotic stress that negatively affects normal plant growth and development by causing tissue injury and delayed growth (Chinnusamy *et al.* 2007). There is a substantial evidence for the involvement of miRNA-based regulatory mechanisms in a wide range of biological processes including plant developmental and abiotic and biotic stress responses (Kidner and Martinssen, 2005; Rubio-Somoza and Weigel, 2011; Chen *et al.* 2012; Cao *et al.* 2014; Megha *et al.* 2017). Although an increasing number of canola miRNAs have been identified under various developmental stages and environmental conditions, including during seed development and under cadmium stress (Huang *et al.* 2010; Korbes *et al.* 2012; Zhou *et al.* 2012; Huang *et al.* 2013; Cheng *et al.* 2017; Wang *et al.* 2017); little is known about the roles of miRNAs involved in cold responses in canola. This study presents, for the first time, a comprehensive analysis of sRNA populations in *B. napus* tissues in response to CS, identified through high throughput sequencing. In addition, a variety of physiochemical changes in response to CS were also monitored in *B. napus*, and the expression of target genes of key candidate miRNAs were compared in the spring and winter variety canola.

Cell membranes are early targets of CS and are subject to its negative effects including an increase in permeability as a result of decrease in integrity (Zhang *et al.* 2017). The relative EL of CS plants increased gradually with duration of cold treatment providing a clear evidence for the loss of membrane integrity after cold exposure. MDA is the final lipid peroxidation product in plant cell membrane and, thus is an important indicator reflecting membrane damage by chilling stress (Taulavuori *et al.* 2001). For example,

increased MDA was observed in rice, sandalwood, coffee and oats under chilling stress (Huang and Guo, 2005; Zhang *et al.* 2017; Campos *et al.* 2003; Liu *et al.* 2013). In this experiment, the enhanced lipid peroxidation until 24 h of CS may be a consequence of increased reactive oxygen species (ROS) production. The decline in MDA level at 48 h of CS could possibly be associated with increase in unsaturated fatty acids (UFAs); while the steady increase in EL observed after CS could be attributed to the direct effects of CS which may be working independently from CS-induced lipid peroxidation (Heidarvand and Maali-Amiri, 2013).

Under adverse conditions such as chilling, freezing, high temperature etc., generation of ROS superoxide, hydrogen peroxide, and hydroxyradicals is enhanced, thereby disturbing the normal redox environment of cells (Foyer and Noctor 2003; Apel and Hirt, 2004). Elevated ROS levels can damage cellular structures and macromolecules eventually leading to cell death (Krasenky and Jonak, 2012). Plants have developed antioxidant defense system against damage from oxidative stress (Wang *et al.* 2016). CAT and POD enzymes can scavenge $H_2O_2$ produced under CS and help protect membrane systems (Liu *et al.* 2013). The increased activities of CAT and POD after 1 h of CS may be important in canola cold tolerance, providing one of the first lines of defense against deleterious effects of elevated ROS levels (Figure 2.2a). A fine balance between CAT and POD activities is crucial for containing toxic ROS levels in a cell and slight alterations in this balance is capable of inducing compensatory mechanisms (Apel and Hirt, 2004). Decreased levels of both antioxidant enzymes at 48 h of CS indicate the possibility of increased cell injuries which may trigger a secondary response to stress conditions.

Low temperature is one of the most important factors that influence plant photosynthesis and decreases the utilization of light. Cold stress disrupts key processes of photosynthesis, including thylakoid electron transport, carbon assimilation and stomatal control (Allen and Ort, 2001). Results from this study confirmed that endogenous chlorophyll and carotenoid contents were both negatively affected by CS and this reduction could be viewed as a typical symptom of oxidative stress by CS treatment (Figure 2.2a). Reduced chlorophyll a / b and carotenoid content have also been observed in rice, wheat and oats subjected to CS (Habibi *et al.* 2011; Aghaee *et al.* 2011; Liu *et al.* 2013).

Using high throughput sRNA sequencing, we obtained 2-4 million unique sRNA reads per sample, which provided adequate sequencing depth for further analysis. In plants, the large majority of sRNAs are 21 and 24 nt in length (Axtell and Bartel, 2005), and the fraction of miRNAs varies among different plant species and environmental conditions (Wei *et al.* 2009; Ding *et al.* 2015; Xu *et al.* 2016). In our study, investigation of the length distribution of sRNA sequences showed a prevalence of 24 nt sRNA, with an average occurrence of 42.25%, followed by 21 nt sRNA species (average occurrence of 29.83%) (Fig. S1). These sRNAs are typically the products of dicer activity and our results are consistent with previous reports in *Arachis hypogea* (Chi *et al.* 2011)*, B. napus* (Huang *et al.* 2013), *B. oleracea* (Wang *et al.* 2012), *Citrus sinensis* (Lu *et al.* 2014), *Solanum lycopersicum* (Cao *et al.* 2014) and *Glycine max* (Xu *et al.* 2016) in which the sRNA transcriptome was dominated by 24 nt sRNAs. The class of 24 nt sRNAs are generally associated with guiding DNA methylation and heterochromatin formation (Lipmann and Martienssen, 2004; Jones-Rhodes and Bartel, 2004, Dolgosheina *et al.* 2008). The 5’

terminal nucleotide of a miRNA redirects it into different AGO complex thereby altering its biological activity (Mi *et al.* 2008). Moreover, uracil serves as the dominant base at first nucleotide position in mature miRNA which determines its loading by AGO1 complex (Zhang *et al.* 2009). In contrast, mature miRNAs with adenine as their first base are associated to AGO2 (a protein of unknown function) and AGO4 (which controls DNA methylation and transcriptional gene silencing) (Zilberman *et al.* 2007; Mi *et al.* 2008). Similar trends have also been observed in other studies where first position of mature miRNAs is represented with adenine (Rubio-Somoza *et al.* 2009; Voinnet, 2009; Czech and Hannon, 2011; Zhao *et al.* 2012; Zhang *et al.* 2014a). Therefore, the predominance of one type of base at 5' terminal of mature miRNAs might be involved in remodelling of 5' end binding pocket of AGO complexes, thereby helping in downstream assortment of sRNA sequences into different AGO complexes (Mi *et al.* 2008).

In the present study, 25 out of 70 conserved miRNAs were identified as CS-responsive and the vast majority of them showed a reduced expression level (Supplementary File 5). This is in agreement with previous research in *Brachypodium*, tomato and soybean where a large percentage of detected miRNAs were reported to be CS-suppressed (Zhang *et al.* 2009; Cao *et al.* 2015; Xu *et al.* 2016). No consistent regulatory pattern was observed for members of miRNA families (miR168, miR394, miR395 and miR398), suggesting possible different functions of miRNAs from the same family (Supplementary File 5). Similar results have been reported in CS tea (Zhang *et al.* 2014a). In addition, high abundance for miR166 and miR159 observed in this study also has been reported in other plant species, including cotton (Yang *et al.* 2013), banana (Bi *et al.* 2015),

radish (Nie *et al.* 2015), *Catharanthus roseus* (Shen *et al.* 2017) and wheat (Song *et al.* 2017) (Figure 4a). Of the miRNAs showing significantly altered expression in canola, several chilling-responsive miRNAs are conserved among several plant species. Consistent with the earlier studies where miR395 was induced by chilling stress in tomato, *Populus tomentosa* and *A. thaliana* (Sunkar and Zhu, 2004; Chen *et al.* 2012; Cao *et al.* 2015), we observed the induction of miR395 by CS, indicating that some miRNAs show similar response among plant species. In addition, some miRNAs such as miR393 which were induced under CS in *A. thaliana* also showed an upward trend with increasing duration of stress in this study (Sunkar and Zhu 2004; Liu *et al.* 2008). Several previously reported chilling-responsive miRNAs, such as miR169, miR319 and miR396 in *A. thaliana* (Sunkar and Zhu, 2004), sugarcane (Thiebaut *et al.* 2012), tomato (Cao *et al.* 2014) and soybean (Xu *et al.* 2016) were not detected in the present study, suggesting that these miRNAs may be species-specific and their expression was not altered in canola after CS, or perhaps the altered expression of above-mentioned miRNAs did not occur during the duration or developmental stage of canola plants, used in this study.

We found that the targets of both novel and conserved cold-responsive miRNAs were often associated with development and other abiotic stresses such as heat, salt and drought (Supplementary File 7 and 8). For example, miR164 targets family of transcription factor *NAC* (for *NAM/ATAF1, 2 / CUC2)* which mediates shoot and root development, flowering time and is also involved in response to cold, drought, salinity, and submergence (Hu *et al.* 2006, 2008; Jeong *et al.* 2010; Hasson *et al.* 2011; Nuruzzaman *et al.* 2012). The loss of a NAC-domain TF, *LOV1 (Long Vegetative Phase 1)*, results in hypersensitivity to

cold, whereas a gain-of-function allele conferred cold tolerance in *A. thaliana* (Yoo *et al.* 2007). Moreover, *LOV1* has been shown to function as a floral repressor suggesting that *LOV1* acts as a common regulator of pathways controlling CS response and flowering time (Yoo *et al.* 2007).

One of the conserved miRNA in plants, miR393, has been observed in different plant species (Navarro *et al.* 2006). In *Brassica*, the targets of miR393 were found to be F-box genes encoding auxin receptors (Transport Inhibitor Response Protein1 (TIR1), Auxin Signaling F Box Protein (AFB3) and GRR-like protein 1 (AFB1)). In *A. thaliana,* miR393–TIR1/ AFB3 regulatory network is known to have multiple functions that manipulate the auxin responses such as controlling the root architecture, regulating leaf development and responses to abiotic and biotic stresses (Chen *et al.* 2011; Si-Ammour *et al.* 2011;Vidal *et al.* 2010; Windels *et al.* 2014; Sunkar and Zhu 2004, Navarro *et al.* 2006). The expression of *TIR1*, *AFB3* and *GRR1* was repressed in 'DH12075' and, as all three of these genes are positive regulators of auxin signalling, their degradation by increased miR393 levels may down-regulate auxin signalling pathway which may inhibit plant growth and development under CS (Sunkar and Zhu, 2004; Rahman, 2013). Similarly, miR394 also targets F-box protein which also acts as auxin receptor and might be involved in auxin modulation under CS. Although it has been reported that CS results in reduced shootward transport of auxin and diminishes the root's capability to form an auxin gradient (Shibaski *et al.* 2009), it would be intriguing to know how a decrease in auxin concentration enhances or diminishes plant's response towards chilling stress.

Among the cold- responsive miRNAs, miR395 was found to be induced over the duration of CS (Figure 2.6a). ATP sulfurylases (*APS1*, *APS3* and *APS4*) are ubiquitous enzymes that catalyze the primary step of intracellular sulfate activation and are predicted targets of miR395 (Jones-Rhoades and Bartel, 2004; Kawashima *et al.* 2009). In *Glycine max,* expression of *APS* gene was up-regulated in response to low temperature (Phartiyal *et al.* 2006). Over-expression of *APS* resulted in enrichment of glutathione – one of the three most abundant antioxidants in plant cells (Kopriva *et al.* 2001). Increased levels of glutathione have resulted in enhanced cold tolerance of apple by maintaining reduced cellular redox environment in cell *via* metabolizing various ROS (Kocsy *et al.* 2004; Wang *et al.* 2016). Since APS is the key rate-limiting enzyme of sulfur assimilatory pathway, expecting an enhanced expression of its transcript under CS is rational. Our results showed that CS increased the expression of *APS4* transcript but decreased the expression of transcripts of *APS1* and *APS3*. The increased expression of miR395 followed by a subsequent up-regulation of *APS4* transcript might be important for mediating plant responses to CS in both 'DH12075' and 'Mendel'.

Heat shock proteins (HSPs) are a group of proteins induced by environmental stress either to protect the plant from damage caused by the stress (through membrane protection, maintaining proteins in their functional conformations) or to help repair the damage caused by the stress (the refolding of denatured proteins and preventing protein aggregation) (Timperio *et al.* 2008). bna-N_miR12 was predicted to target the transcript of HSP by psRNA Target. While the expression of bna-N_miR12 was lower in 'DH12075' and higher in 'Mendel' after CS, the expression of *HSP* was found to be induced by CS in

plants. The decreased expression of *HSP* transcript after CS treatment in both 'DH12075' and 'Mendel' can be suggestive of the fact that HSP proteins are not involved in protection of plant under chilling treatment at the time points tested in this study. This observation could further suggest that the shared regulatory genes and networks induced by the stress-induced miRNAs might be involved in diverse stress responses.

### ***RAV1*, *MYC-C* and LTRE elements regulate CS response in *B. napus***

*Cis*-acting elements are involved in regulation of gene activities controlling various processes, such as response to abiotic and biotic stress, and hormones and have been analyzed extensively (Kasuga *et al.* 1999, Zhang *et al.* 2005; Liu *et al.* 2008). *In silico* analysis of cis-acting elements in promoter region of plant miRNA genes have been conducted previously to elucidate miRNA-mediated gene regulation (Liu *et al.* 2008; Zhou *et al.* 2008; Zhao *et al.* 2012; Zhang *et al.* 2014b). Analysis of 14 miRNAs indicated the presence of ABREs in their putative promoters suggesting that these miRNAs might be involved in ABA-mediated cold response. Additional evidence for the involvement of hormones in cold defence system mediated via miRNAs comes from the presence of phytohormone-regulated elements such as MeJA-responsiveness element and GARE (GA-responsive element) at 5' of eight and nine miRNAs, respectively (Table 2.1) indicating that these miRNAs might regulate CS response via modulation of levels of these hormones.

Previously, *RAV1* (related to ABI3/VP1) TF was identified by microarray analysis of cold stressed *A. thaliana* (Fowler and Thomashow, 2002). The expression of *RAV1* induces in parallel with the *CBF* regulon (Fowler *et al.* 2005) and thus the presence of *RAV1* binding sites in all of 14 miRNA genes indicate the possible role of these elements in

CS signalling pathways. MYC (CANNTG) is the binding site of *ICE1* and was the second most abundant class found in the present study. Cold stress-activated *ICE1* acts upstream of the *CBF3* by binding to the MYC recognition sequences on *CBF3* promoter thereby, inducing its expression and regulation of target *COR* genes (Chinnusamy *et al.* 2004; Chinnusamy *et al.* 2010; Theocharis *et al.* 2012). MYC cis-element has also been shown to be involved in chilling response in *Triticum aestivum* and *Paeonia suffruticosa* (Zhang *et al.* 2016; Song *et al.* 2017). Both *RAV1* and MYC elements were also identified in soybean miR166u, miR171p, miR2111f and miR169c and their target genes (*e.g. glyma08g21620*, *glyma07g01940*) (Zhang *et al.* 2014b).

Among the 14 miRNA genes analyzed, 10 miRNAs contained LTRE repeats (Table 2.1). The presence of LTRE repeats in the promoter region of LT responsive gene, *Bn115* has been implicated in LT regulation of *Bn115* (White *et al.* 1994). LTRE repeats have also been reported in cold-responsive miRNAs such as, miR156k, miR166m, miR168b in rice, miR156h, miR168, miR397 in *A. thaliana*, and in soybean miR169c (Liu *et al.* 2008; Lv *et al.* 2010; Zhang *et al.* 2014b). Consistent with previous studies, we also observed the presence of LTRE repeats in miR168 and miR397 (Table 2.1). Therefore, we can conclude that these cis-elements are involved in regulation of gene expression involved in CS at both transcriptional and post-transcriptional level.

**Conclusion**

The present study generated a well annotated miRNAome for the CS response in *B. napus*. Based on the measurement of physiological parameters, sRNA sequencing and

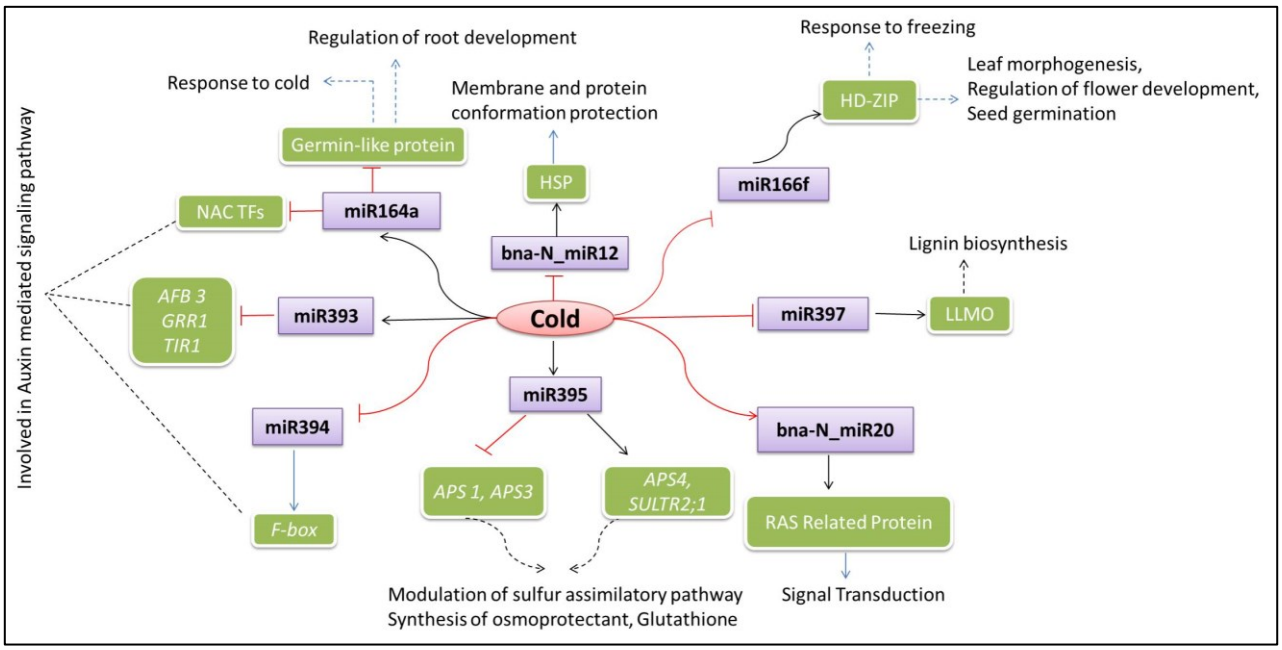


**Figure 2.8:** Regulatory network of chilling responsive miRNA and their targets. Red indicates down-regulation and black indicates up-regulation.

qRT-PCR analysis, we have provided evidence for the involvement of miRNA-target gene regulatory networks and related physiological changes in canola when exposed to CS. Upon exposure to CS, cold signal transduction induces changes in cell membrane (determined by electrolyte leakage and MDA content) and leads to accumulation of antioxidant enzymes such as POD and CAT. Photosynthetic efficiency and chlorophyll content is negatively affected by CS. Prediction and analysis of target genes for these cold response miRNAs has demonstrated that the involvement of numerous TFs, hormone signalling genes, ROS signalling network as well as genes affecting plant growth and development. Phytohoromone, auxin might play an important role in canola response to CS and needs to be investigated. Detection of cold-responsive miRNAs, which have been

reported in response to other abiotic stresses, suggests cross regulation of miRNAs between different pathways in plant responses to various stresses. In addition, some miRNAs and their targets have been found to be common players in flowering and CS response. An increased expression of CBF genes indicates that CBF-dependent signalling pathway plays an important role in regulation of CS response in *B. napus*. A hypothetical model for canola CS response has been proposed (Figure 2.8). Taken together, this comprehensive study provides the first global CS responsive miRNA expression profile in *B. napus* and these findings lay the foundation for exploring the complex miRNA-mediated regulatory networks in plant response to CS and other abiotic / biotic stresses.

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# Supplementary Files

**Supplementary File 1**: List of primers used in this study.

| | |
|---|---|
| CBF5_F | GAGCTGTCCGAAGAAACCTG |
| CBF5_R | ATCTCGGCGGTTAGGAAAGT |
| CBF17_F | CTGGACATGGAGGAGACGAT |
| CBF17_R | GCCATATCAGCCAACAAGGT |
| Bn115_F | CGACGGAGAAGACAAAGGAG |
| Bn115_R | GCAACTTTGTTCCCAGCTTC |
| COR25_F | GATCGTGGCTTGTTCGATTT |
| COR25_R | GGAGCTATTGGATCGGTGAA |
| miR166_F | TCGGACCAGGCTTCATTCCCC |
| miR168_F | TCGCTTGGTGCAGGTCGAGAA |
| miR393_F | TCCAAAGGGATCGCATTGATC |
| miR394_F | TTGGCATTCTGTCCACCTCC |
| miR395_F | CTGAAGTGTTTGGGGGGACTC |
| miR397_F | TCATTGAGTGCAGCGTTGATGT |
| Bna_N_miR12_F | TCGATAAACCTCTGCATCCAG |
| Bna_N_miR20_F | ATTTTTCGGCTAAGAGACGGTTCT |
| HDZIP_F | GGCATCGTCGCTGTTTCAC |
| HDZIP_R | CGACAGTCACGGAACCAAGA |
| RRM_F | AGCGAGACAGAACGGTCCAA |
| RRM_R | TCCGGAGCCATAGTGACAATG |
| GRR1_F | GAGAACGCGGCCAAACTAGA |
| GRR1_R | GCTTTGGCATTTTTGGACTCA |
| TRR1_F | ACAAGCTTGGAAGGTGTTGGA |
| TRR1_R | TGCGGCGAGATTTGTGATT |
| FBOX_F | GGACCATCCCGTCCAACATA |
| FBOX_R | TCGGGCTCCGTTAACATGA |
| LLMO_F | ATGCCTAAAATCGCGTTGCT |
| LLMO_R | GTGGCCATGTTCGTGACAGA |
| HSP_F | GGCAGGCGGTTGTTAATCC |
| HSP_R | CCCGTTATCATCCTTCACAACTC |
| RAS_F | GTGTCTGAATCACACACGAAACAC |
| RAS_R | CTCTCCGGCGAGACGACAT |
| APS1_F | AGCGAAGGCTGGGCAAGT |
| APS1_R | AGGACGATAGGCACCGACAT |
| APS3_F | TGAAACTTACGGCGATCGATT |
| APS3_R | GAGACGAAGCGAATTGAAATGA |
| APS4_F | CCGAGGCTTCATGAGACAGT |
| APS4_R | GGGGTTACCAGACGAGTCAA |
| UBC_F | GGACCATCCCGTCCAACATA |
| UBC_R | TGAAACTTACGGCGATCGATT |
| Gene Racer 5′ Primer | CGACTGGAGCACGAGGACACTGA |
| Gene Racer 5′ Nested p | GGACACTGACATGGACTGAAGGAC |
| ASP1_GSP1_Race | GAAAGCCTGAAACGGTCAAG |
| ASP1_GSP2_Race | TGTTCTGGCTATCCGCTCTT |
| ASP3_GSP1_Race | GGCGAGTGTCAGTCATGAGA |
| ASP3_GSP2_Race | ACCACCAATGAGCCAGTTTC |
| ASP4_GSP1_Race | TGTGTACGGGGTTCCTTAGC |
| ASP4_GSP2_Race | CTGCGTAAGGAAGACCTGGA |
| SULTR2;1_GSP1_Race | CGTGCCCCACGTTCTAGCTATTC |
| SULTR2;1_GSP2_Race | ACCATCCTCAAGCACCTTCTCGT |
| ISP_GSP1_Race | CCACCAGTGCAAGAAATCCT |
| ISP_GSP2_Race | GAATCGGTCCAGATCGTAGC |
| HSP_GSP1_Race | GCAGCAAACTGTTTCCCAAT |
| HSP_GSP2_Race | AGCAGGCACAGTGACAACAG |
| F_box_GSP1_Race | TCCCGCAAGCGATCCAAAGTCTCTT |
| F_box_GSP2_Race | CAGCATCGTACATTATCAGACGGTT |
| ABF3_GSP1_Race | AACAGAATCCGTTCTTGTCTGAA |
| ABF3_GSP2_Race | AAACAAAACCAACACGGAGACT |
| GRR1_GSP1_Race | CCAACAATTTTGGTGAAGACAA |
| GRR1_GSP2_Race | ATCTTGGTCCTGCGAGTGTC |
| TIR1_GSP1_Race | TACCCCACCAGAATCTCTCAGT |
| TIR1_GSP2_Race | GCAAACCCTCATGGTTGAGT |

**Supplementary File 2**: Small RNA mapping information using a composite genome constituted of *B. rapa,* GSS sequences of *B. napus* and *B. oleracea* as reference.

| | | Total raw reads | Clipped reads | Mapable reads |
|---|---|---|---|---|
| 0 h | BR_1 | 4,043,379 | 3,587,264 | 635,091 |
| | BR_2 | 3,529,740 | 3,051,960 | 558,379 |
| | BR_3 | 5,850,705 | 4,888,034 | 760,376 |
| 1 h | BR_1 | 5,957,430 | 5,384,823 | 952,087 |
| | BR_2 | 5,394,780 | 4,603,540 | 801,322 |
| | BR_3 | 4,061,179 | 3,510,291 | 656,032 |
| 2 h | BR_1 | 5,375,806 | 4,715,780 | 887,630 |
| | BR_2 | 4,853,178 | 4,285,541 | 909,351 |
| | BR_3 | 8,586,984 | 7,385,836 | 1,169,704 |
| 4 h | BR_1 | 7,022,118 | 6,172,925 | 1,156,894 |
| | BR_2 | 5,890,626 | 5,043,476 | 920,326 |
| | BR_3 | 1,627,234 | 1,270,889 | 156,106 |
| 8 h | BR_1 | 39,561,148 | 27,607,050 | 2,372,764 |
| | BR_2 | 4,410,526 | 5,225,346 | 974,931 |
| | BR_3 | 6,319,785 | 5,176,705 | 754,802 |
| 24 h | BR_1 | 7,807,581 | 6,667,219 | 1,236,285 |
| | BR_2 | 6,272,018 | 5,110,580 | 887,156 |
| | BR_3 | 7,474,508 | 5,991,657 | 849,228 |
| 48 h | BR_1 | 3,988,640 | 3,371,890 | 603,778 |
| | BR_2 | 10,237,012 | 7,852,664 | 1,081,517 |
| | BR_3 | 5,440,874 | 4,418,670 | 657,752 |

**Supplementary File 3:** List of novel miRNAs identified in this study with precursor information and counts per million (CPM).

| | | | | | | | CPM | CPM | CPM | CPM | CPM | CPM | CPM | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sequence | Length o | Precurso | Length o | MFE (kc | MFEI | 0h (contr | 1h | 2h | 4h | 8h | 24h | 48h | |
| bna-N_miR1 | cagagaguaagacauaguagaaga | 24 | uucuacuau | 68 | -45.10 | -1.67 | 11.06 | 12.13 | 16.69 | 0.22 | 0.22 | 0.22 | 0.22 | 40.77 |
| bna-N_miR2 | agugagaaauggagugaugaaca | 23 | gagugagaa | 69 | -37.90 | -1.58 | 44.78 | 45.08 | 33.15 | 66.76 | 0.22 | 0.22 | 0.22 | 190.44 |
| bna-N_miR3 | auuccgauaagaacuccaccuua | 23 | agguggagu | 87 | -54.30 | -1.87 | 29.13 | 34.10 | 21.39 | 48.13 | 0.22 | 0.22 | 0.22 | 133.41 |
| bna-N_miR4 | augcgcggagugaguucuuauaau | 24 | uauaggaua | 93 | -37.20 | -1.03 | 0.22 | 8.46 | 8.06 | 8.21 | 0.22 | 0.22 | 0.22 | 25.63 |
| bna-N_miR5 | auaaacuguuaacguaugucaccc | 24 | auaaacugu | 73 | -34.30 | -1.27 | 0.22 | 13.04 | 22.17 | 11.76 | 9.00 | 0.22 | 0.22 | 56.64 |
| bna-N_miR6 | uggagaagcagagcacgugca | 21 | uggagaagc | 77 | -33.00 | -0.92 | 23.10 | 51.49 | 44.13 | 49.90 | 8.12 | 36.03 | 51.23 | 264.01 |
| bna-N_miR7 | cggaaaauuaaugcacugcac | 21 | ucagugugu | 81 | -33.00 | -1.03 | 44.78 | 35.93 | 35.50 | 11.76 | 20.40 | 57.66 | 37.50 | 243.53 |
| bna-N_miR8 | cuugcauaucuuaggagcuuu | 21 | agcugcuaa | 90 | -30.40 | -0.89 | 566.23 | 628.28 | 256.58 | 28.61 | 418.72 | 764.10 | 1255.83 | 3918.34 |
| bna-N_miR9 | agcugcuaagcuaugaauccc | 21 | agcugcuaa | 85 | -25.80 | -0.86 | 104.99 | 215.37 | 300.48 | 254.82 | 259.92 | 437.36 | 475.98 | 2048.93 |
| bna-N_miR10 | agcagaauauaagaacccgacucu | 24 | agcagaaua | 70 | -27.40 | -1.19 | 59.23 | 63.40 | 143.69 | 105.79 | 153.76 | 48.71 | 85.57 | 660.14 |
| bna-N_miR11 | auauuuuuuggaccaaguuaagcu | 24 | cuggggcuu | 80 | -28.80 | -0.65 | 0.22 | 18.53 | 11.20 | 29.50 | 16.89 | 13.65 | 22.79 | 112.78 |
| bna-N_miR12 | ugauuaacccggaguucuuag | 21 | agagacggg | 102 | -65.30 | -1.48 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 21.80 | 23.15 |
| bna-N_miR13 | agcagaauauaagaacccgucucu | 24 | uuuuuauau | 92 | -38.70 | -1.07 | 0.22 | 64.31 | 52.75 | 122.64 | 69.53 | 36.78 | 152.27 | 498.50 |
| bna-N_miR14 | uugaaagucgucuaugauaag | 21 | uugaaaguc | 85 | -30.70 | -0.96 | 0.22 | 25.86 | 16.69 | 19.74 | 13.38 | 19.62 | 0.22 | 95.73 |
| bna-N_miR15 | aaguagaauucucuagauuuaaga | 24 | uaaaaaauc | 88 | -22.60 | -1.51 | 68.87 | 0.22 | 0.22 | 87.16 | 71.29 | 74.07 | 147.36 | 449.20 |
| bna-N_miR16 | agagauuuuuguuacuguuaac | 22 | uucuaacag | 63 | -16.20 | -1.25 | 71.28 | 0.22 | 0.22 | 0.22 | 78.31 | 56.92 | 89.49 | 296.66 |
| bna-N_miR17 | auuuuucggcuaagagacgguucu | 24 | auuuuucgg | 84 | -29.20 | -1.46 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.92 | 11.01 | 22.05 |
| bna-N_miR18 | uaguggaagcagcaacgagaa | 21 | cuuuuugcu | 88 | -33.90 | -1.00 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.92 | 17.88 | 28.92 |
| bna-N_miR19 | acgagucauacuccgucggua | 21 | ccgacgaau | 80 | -41.60 | -1.01 | 13.47 | 0.22 | 51.18 | 27.72 | 37.95 | 25.59 | 30.63 | 186.77 |
| bna-N_miR20 | auccggauccguaaguuuuauua | 23 | guaaaauug | 68 | -33.00 | -1.32 | 23.10 | 35.01 | 0.22 | 36.59 | 57.25 | 30.06 | 72.81 | 255.06 |
| bna-N_miR21 | accggaaguuaagaaacuguuucu | 24 | accggaagu | 74 | -22.30 | -1.12 | 107.40 | 56.99 | 0.22 | 0.22 | 116.03 | 90.49 | 148.35 | 519.70 |
| bna-N_miR22 | gucggccgggggacggacugggaa | 24 | cccaguucc | 96 | -59.70 | -0.90 | 3149.37 | 2998.58 | 7088.76 | 0.22 | 4730.05 | 2341.07 | 5143.29 | 25451.35 |
| bna-N_miR23 | auauaugucaaccaaucgagcuuu | 24 | aucguuggu | 77 | -25.20 | -0.90 | 0.22 | 0.22 | 0.22 | 19.74 | 15.14 | 17.38 | 0.22 | 53.15 |
| bna-N_miR24 | agcggaugagaucugcaugcaga | 23 | agcggauga | 86 | -35.60 | -1.02 | 14.67 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 16.02 |
| bna-N_miR25 | aucugaauggaucaucugaauaga | 24 | uuagaugau | 78 | -21.90 | -0.84 | 24.31 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 25.65 |
| bna-N_miR26 | auuauccgcggauauccggauccg | 24 | uccgucgga | 93 | -44.00 | -0.96 | 19.49 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 20.83 |
| bna-N_miR27 | cccgucucuuaacuuuuaacuaaa | 24 | cccgucucu | 99 | -53.90 | -1.74 | 21.90 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 23.24 |
| bna-N_miR28 | gcaaguagacuuuggcucugu | 21 | ugagccaaa | 82 | -38.50 | -1.13 | 23.10 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 24.45 |
| bna-N_miR29 | gcaagucgacuuuggcucugu | 21 | ugagccaaa | 79 | -35.00 | -1.09 | 13.47 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 14.81 |
| bna-N_miR30 | uacucggacgacuuacauuucagu | 24 | uacucggac | 74 | -34.10 | -1.18 | 68.87 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 70.21 |
| bna-N_miR31 | ucuuaacuucgaauaaaagacggu | 24 | ucuuaacuu | 93 | -24.60 | -1.07 | 5.04 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 6.38 |
| bna-N_miR32 | uuaauuaaaaguuaagaggcgguu | 24 | ccgucucuu | 74 | -27.50 | -1.31 | 6.24 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 7.59 |
| bna-N_miR33 | uuggaguccuuaaguauauuaua | 23 | aaauauacu | 60 | -26.70 | -1.27 | 5.04 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 6.38 |
| bna-N_miR34 | aagaugucgucggaauauuccga | 23 | aauauuccg | 73 | -34.40 | -1.15 | 0.22 | 9.38 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 10.72 |
| bna-N_miR35 | aaguuuaagucgucugguaccag | 23 | gucuggacg | 60 | -24.20 | -0.86 | 0.22 | 9.38 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 10.72 |
| bna-N_miR36 | aauauauaagauggauggggcc | 21 | aauauauaa | 72 | -25.20 | -0.97 | 0.22 | 11.21 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 12.55 |
| bna-N_miR37 | acacaugaaucaaaguugugacuu | 24 | acacaugaa | 65 | -32.20 | -1.61 | 0.22 | 46.92 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 48.26 |
| bna-N_miR38 | auauacuaaagcacaagucacaug | 24 | auauacuaa | 63 | -18.20 | -0.91 | 0.22 | 67.06 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 68.40 |
| bna-N_miR39 | auuuaaaauuccgaggaaaugaacc | 24 | auuuaaaau | 90 | -46.90 | -1.20 | 0.22 | 10.29 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 11.64 |
| bna-N_miR40 | cggauccggauguuaaauguua | 23 | aggcauggg | 90 | -39.20 | -0.93 | 0.22 | 9.38 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 10.72 |
| bna-N_miR41 | cuaaaaaagcuaagagacauc | 21 | cuaaaaaag | 66 | -26.50 | -1.56 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 2.19 | 3.53 |
| bna-N_miR42 | cuuaccugagaagucgucuggacg | 24 | gaccagacg | 64 | -43.30 | -1.27 | 0.22 | 6.63 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 7.97 |
| bna-N_miR43 | gaaaaaugcagucaugaacaaac | 23 | gaaaaaugc | 89 | -39.20 | -1.57 | 0.22 | 8.46 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.81 |
| bna-N_miR44 | gaauaugucgucggaauauuccga | 24 | aauauuccg | 72 | -36.00 | -1.24 | 0.22 | 11.21 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 12.55 |
| bna-N_miR45 | auucggauucgguuuggacggauc | 24 | aauguguuu | 85 | -34.70 | -1.05 | 0.22 | 8.46 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.81 |
| bna-N_miR46 | ugcuccaauguauaccucuauaau | 24 | ugcuccaau | 63 | -20.00 | -1.11 | 0.22 | 20.37 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 21.71 |
| bna-N_miR47 | uguaagagcaucuccaaccccacu | 24 | uguaagagc | 77 | -25.40 | -0.85 | 0.22 | 14.87 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 16.21 |
| bna-N_miR48 | uucaaguaagucgucugggua | 21 | gccuggacg | 61 | -33.10 | -1.07 | 0.22 | 27.69 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 29.03 |
| bna-N_miR49 | uuguucaucacuccauuucccacu | 24 | agugggaaa | 91 | -52.70 | -1.88 | 0.22 | 11.21 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 12.55 |
| bna-N_miR50 | uuuaacuaaaaaaacuaagaaccg | 24 | uuuaacuaa | 70 | -34.50 | -2.16 | 0.22 | 13.96 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 15.30 |
| bna-N_miR51 | uuuguucaugacuguauuuuccacu | 25 | gaaaaaugc | 89 | -39.20 | -1.57 | 0.22 | 10.29 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 11.64 |
| bna-N_miR52 | aauaacaguggauuuuaggguacu | 24 | acaccccaa | 76 | -17.70 | -0.88 | 0.22 | 0.22 | 14.33 | 0.22 | 0.22 | 0.22 | 0.22 | 15.68 |
| bna-N_miR53 | agaguagaguucuuagcggaaguu | 24 | aacuuccgc | 91 | -41.10 | -1.11 | 0.22 | 0.22 | 30.01 | 0.22 | 0.22 | 0.22 | 0.22 | 31.36 |
| bna-N_miR54 | auaagagauauaagaaccgguucu | 24 | gaaccguuu | 73 | -20.80 | -0.95 | 0.22 | 0.22 | 44.91 | 0.22 | 0.22 | 0.22 | 0.22 | 46.25 |
| bna-N_miR55 | auagagugaaauauagaggaaugu | 24 | auuugcucc | 65 | -18.30 | -0.91 | 0.22 | 0.22 | 19.04 | 0.22 | 0.22 | 2.46 | 0.22 | 22.62 |
| bna-N_miR56 | caucuucacccagauggaucaccu | 24 | gggugaucc | 76 | -30.80 | -0.93 | 0.22 | 0.22 | 16.69 | 0.22 | 0.22 | 0.22 | 0.22 | 18.03 |
| bna-N_miR57 | cucgucucuuaacuuuuaacuaaa | 24 | cucgucucu | 85 | -32.40 | -1.47 | 0.22 | 0.22 | 11.98 | 0.22 | 0.22 | 0.22 | 0.22 | 13.32 |
| bna-N_miR58 | gggaagucgucuggcuguagacga | 24 | gggaagucg | 63 | -30.00 | -0.99 | 0.22 | 0.22 | 8.06 | 0.22 | 0.22 | 0.22 | 0.22 | 9.41 |
| bna-N_miR59 | guaagucguccaguuggaaaaccu | 24 | uuccagacg | 73 | -31.60 | -1.09 | 0.22 | 0.22 | 15.12 | 0.22 | 0.22 | 0.22 | 0.22 | 16.46 |
| bna-N_miR60 | guugcgcggaucgagaucuagu | 22 | guugcgcgg | 86 | -32.70 | -1.02 | 0.22 | 0.22 | 0.22 | 0.22 | 19.53 | 0.22 | 0.22 | 20.87 |
| bna-N_miR61 | guuucgcagacgcugacgcug | 21 | cagcgucag | 72 | -47.40 | -1.03 | 0.22 | 0.22 | 8.85 | 0.22 | 0.22 | 0.22 | 0.22 | 10.19 |
| bna-N_miR62 | uaagaaccggcucuuaaauaaga | 23 | uaagaaccg | 82 | -25.60 | -0.98 | 0.22 | 0.22 | 10.42 | 0.22 | 0.22 | 0.22 | 0.22 | 11.76 |
| bna-N_miR63 | uauccccggacgaccuuaaauu | 21 | uaaagucgu | 85 | -42.40 | -1.18 | 0.22 | 0.22 | 72.35 | 0.22 | 0.22 | 0.22 | 0.22 | 73.69 |
| bna-N_miR64 | uauccuggaugacuuucaagu | 21 | aagaaaguc | 87 | -32.90 | -1.00 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 22.79 | 24.13 |
| bna-N_miR65 | uguaagucguccaguuggaaaac | 23 | uuccagacg | 71 | -31.60 | -1.17 | 0.22 | 0.22 | 8.85 | 0.22 | 0.22 | 0.22 | 0.22 | 10.19 |
| bna-N_miR66 | ugugacuugugcuuuaguauaua | 23 | auacuaaag | 71 | -40.00 | -1.82 | 0.22 | 0.22 | 14.33 | 0.22 | 0.22 | 0.22 | 0.22 | 15.68 |
| bna-N_miR67 | aacucuaaacccuaaacccuaaac | 24 | aacucuaaa | 63 | -20.00 | -0.87 | 0.22 | 0.22 | 0.22 | 82.72 | 0.22 | 0.22 | 0.22 | 84.06 |
| bna-N_miR68 | aagaaccgucucuuaaaauagaga | 24 | aagagacgg | 60 | -26.50 | -1.39 | 0.22 | 0.22 | 0.22 | 11.76 | 0.22 | 0.22 | 0.22 | 13.10 |
| bna-N_miR69 | aagaaccgucucuuaaccgaagcu | 24 | ucuuauuua | 77 | -28.60 | -1.19 | 0.22 | 0.22 | 0.22 | 8.21 | 0.22 | 0.22 | 0.22 | 9.55 |
| bna-N_miR70 | aauuccgacggauaguaaaauauc | 24 | gauacuauc | 83 | -33.00 | -1.06 | 0.22 | 0.22 | 0.22 | 25.06 | 0.22 | 0.22 | 0.22 | 26.40 |
| bna-N_miR71 | acggaauaccgaugacugaucugu | 24 | ucacauggu | 60 | -25.10 | -0.90 | 0.22 | 0.22 | 0.22 | 9.09 | 0.22 | 0.22 | 0.22 | 10.44 |
| bna-N_miR72 | agcugaauauaagaauuugucucu | 24 | agcugaaua | 70 | -15.20 | -0.95 | 0.22 | 0.22 | 0.22 | 10.87 | 0.22 | 0.22 | 0.22 | 12.21 |
| bna-N_miR73 | ccucgcaggauucuucacaauu | 22 | aauugugaa | 84 | -68.00 | -2.19 | 0.22 | 3.89 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 5.23 |
| bna-N_miR74 | uaggguuuaggguuuaggguuu | 22 | acccuaaaa | 90 | -29.90 | -1.07 | 0.22 | 0.22 | 0.22 | 9.09 | 0.22 | 0.22 | 0.22 | 10.44 |
| bna-N_miR75 | uauuccgacggauugauauuuccu | 24 | uaauauauc | 90 | -24.70 | -0.85 | 0.22 | 0.22 | 0.22 | 22.40 | 0.22 | 0.22 | 0.22 | 23.74 |
| bna-N_miR76 | uccucggaauuccgucgguauauu | 24 | uccucggaa | 85 | -37.10 | -0.98 | 0.22 | 0.22 | 0.22 | 26.84 | 0.22 | 0.22 | 0.22 | 28.18 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **bna-N_miR77** | uuguaagucgucucagguuag | 21 | uuuuccugg | 101 | -49.40 | -1.54 | 0.22 | 0.22 | 0.22 | 6.43 | 0.22 | 0.22 | 0.22 | 7.78 |
| **bna-N_miR78** | uuuaggguuuaggguuuaggguuu | 24 | acccuaaau | 90 | -29.90 | -1.07 | 0.22 | 0.22 | 0.22 | 9.09 | 0.22 | 0.22 | 0.22 | 10.44 |
| **bna-N_miR79** | aagaacaaaauagucaacuguua | 23 | aagaacaaa | 79 | -17.20 | -0.86 | 0.22 | 0.22 | 0.22 | 0.22 | 30.93 | 0.22 | 0.22 | 32.27 |
| **bna-N_miR80** | agucaucggaaauaacuccucgg | 23 | agucaucgg | 84 | -34.90 | -1.00 | 0.22 | 0.22 | 0.22 | 0.22 | 9.00 | 0.22 | 0.22 | 10.34 |
| **bna-N_miR81** | auaagaaccccgaguuaaucaugu | 24 | caugauuaa | 77 | -25.50 | -0.85 | 0.22 | 0.22 | 0.22 | 0.22 | 6.37 | 0.22 | 2.19 | 9.67 |
| **bna-N_miR82** | auccggauccgaacuuacuauuua | 24 | uccaaaucc | 79 | -29.10 | -0.86 | 0.22 | 0.22 | 0.22 | 0.22 | 9.87 | 0.22 | 0.22 | 11.22 |
| **bna-N_miR83** | auuaugcaguugugaauaaac | 21 | auuaugcag | 80 | -36.70 | -1.84 | 0.22 | 0.22 | 0.22 | 0.22 | 7.24 | 0.22 | 0.22 | 8.58 |
| **bna-N_miR84** | cucaacuuuaguuccguuaac | 21 | cucaacuuu | 89 | -50.80 | -1.45 | 0.22 | 4.80 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 6.14 |
| **bna-N_miR85** | gaauuccguuggaaaauccug | 21 | gaauuccgu | 62 | -25.00 | -0.89 | 0.22 | 0.22 | 0.22 | 0.22 | 6.37 | 0.22 | 0.22 | 7.71 |
| **bna-N_miR86** | uuagaauacucuuauuuacguaga | 24 | auguagauu | 92 | -19.40 | -0.84 | 0.22 | 0.22 | 0.22 | 0.22 | 12.51 | 0.22 | 0.22 | 13.85 |
| **bna-N_miR87** | uuuagaguuuaggguuuagaguu | 23 | auucgaacc | 89 | -28.80 | -0.93 | 0.22 | 0.22 | 0.22 | 0.22 | 6.37 | 0.22 | 0.22 | 7.71 |
| **bna-N_miR88** | aaccccuaaacucuaaauccuaaac | 24 | uuaggauuu | 73 | -26.40 | -1.26 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.18 | 0.22 | 10.52 |
| **bna-N_miR89** | agaggcauacauuugagcauu | 21 | uuuccaaug | 72 | -27.50 | -1.20 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.18 | 0.22 | 10.52 |
| **bna-N_miR90** | aggacagcuucuucgucguu | 20 | uugggauga | 81 | -29.50 | -0.87 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 47.22 | 53.19 | 101.53 |
| **bna-N_miR91** | agggugaauaugacaaaacaaagu | 24 | agggugaau | 89 | -21.70 | -0.87 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 7.68 | 0.22 | 9.03 |
| **bna-N_miR92** | auauaccgacggacaacggucguc | 24 | auauaccga | 90 | -40.10 | -0.93 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.18 | 0.22 | 10.52 |
| **bna-N_miR93** | auguccaaaaacgcaucgaucgau | 24 | aucgaucga | 110 | -45.80 | -0.97 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 14.40 | 9.05 | 24.57 |
| **bna-N_miR94** | cgcccaaaguucgucggaauaua | 23 | uauuccgac | 87 | -45.40 | -1.16 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 15.14 | 0.22 | 16.48 |
| **bna-N_miR95** | cugaacccuuggauaaaaucauaaa | 24 | cugaacccu | 90 | -42.00 | -1.75 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 27.82 | 0.22 | 29.17 |
| **bna-N_miR96** | gaacgaguaaugaauuuuuggacu | 24 | gaacgagua | 89 | -25.30 | -0.90 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 5.45 | 0.22 | 6.79 |
| **bna-N_miR97** | uacuaaaauaguguagaacuccua | 23 | uacuaaaua | 86 | -58.40 | -2.25 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 5.45 | 0.22 | 6.79 |
| **bna-N_miR98** | uauaaaaucaucguuauuuccuc | 23 | gaggaaauu | 92 | -33.80 | -1.02 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 7.68 | 0.22 | 9.03 |
| **bna-N_miR99** | ucuacaaagucguccaaguagacu | 24 | ucuacaaag | 86 | -50.10 | -1.39 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 56.17 | 0.22 | 57.51 |
| **bna-N_miR100** | uuaguuaaaagcuaagagacc | 21 | ucuauuagc | 98 | -44.10 | -1.58 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.18 | 0.22 | 10.52 |
| **bna-N_miR101** | uuccauguaagucgucuagguaua | 24 | uuccaugua | 73 | -24.00 | -1.14 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 12.16 | 0.22 | 13.50 |
| **bna-N_miR102** | uuuauauuccgcuaagaaccc | 21 | gaguuuuua | 95 | -23.80 | -0.88 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 18.87 | 0.22 | 20.21 |
| **bna-N_miR103** | auauauuacucaaguccccuuucau | 24 | auauauuac | 95 | -37.20 | -1.09 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 36.52 | 37.86 |
| **bna-N_miR104** | auuguccgaugccacgucgacgau | 24 | auuguccga | 62 | -27.90 | -0.90 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 6.11 | 7.45 |
| **bna-N_miR105** | cacauccguuuagaacuccagagu | 24 | cacauccgu | 64 | -24.10 | -0.86 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 13.96 | 15.30 |
| **bna-N_miR106** | cauauauauguauuaaggacc | 21 | cuuaauaca | 88 | -30.40 | -2.03 | 0.22 | 0.22 | 0.22 | 0.22 | 10.75 | 0.22 | 0.22 | 12.09 |
| **bna-N_miR107** | ggaaauuccgaggaaau | 17 | ggaaauucc | 107 | -39.00 | -0.91 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 6.11 | 7.45 |
| **bna-N_miR108** | guuauuuccgaggacuuuuuucgu | 24 | aaaaguccu | 106 | -50.60 | -1.20 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 40.44 | 41.78 |
| **bna-N_miR109** | uaacgguuuauaaguugagggu | 22 | cucaacuuu | 90 | -51.20 | -1.42 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 9.05 | 10.39 |
| **bna-N_miR110** | uaggauuuaggguuuaguguuuu | 23 | aaaccuug | 87 | -23.40 | -0.81 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 6.11 | 7.45 |
| **bna-N_miR111** | ugaacccuuggauaaaucaua | 21 | ugaacccuu | 82 | -41.60 | -1.89 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 27.82 | 0.22 | 29.17 |
| **bna-N_miR112** | uuaucggaauauaagaacccgacu | 24 | uuaucggaa | 69 | -15.60 | -0.87 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 14.94 | 16.28 |
| **bna-N_miR113** | uugcgcggaucgagaucuaguu | 22 | uugcgcgga | 86 | -32.70 | -1.05 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 10.03 | 11.37 |
| **bna-N_miR114** | uuaccggaauauaagaaacugucu | 24 | uuaccggaa | 86 | -21.10 | -0.81 | 70.07 | 42.34 | 48.83 | 40.14 | 36.20 | 44.98 | 58.10 | 340.66 |
| **bna-N_miR115** | uugaaagucguccaugauaag | 21 | uugaaaguc | 87 | -32.70 | -0.99 | 461.46 | 462.57 | 265.20 | 236.19 | 280.98 | 364.26 | 387.70 | 2458.34 |
| **bna-N_miR116** | acgguaucucuccuacguagc | 21 | uucgcagga | 73 | -32.10 | -0.94 | 537.33 | 396.65 | 333.41 | 281.43 | 355.55 | 334.42 | 439.68 | 2678.46 |
| **bna-N_miR117** | agcggaauauaagaaauugucucu | 24 | agcggaaua | 72 | -38.20 | -2.01 | 144.74 | 121.07 | 99.79 | 93.37 | 95.86 | 118.83 | 103.22 | 776.87 |
| **bna-N_miR118** | aacggaauauaagaaccugucucu | 24 | aacggaaua | 72 | -43.30 | -2.06 | 626.44 | 598.06 | 517.64 | 473.93 | 525.76 | 521.66 | 529.93 | 3793.41 |
| **bna-N_miR119** | uuuggacgacuuacauguaagu | 22 | uauuuggac | 69 | -39.20 | -1.51 | 195.31 | 299.60 | 313.81 | 314.25 | 243.25 | 403.79 | 458.32 | 2228.34 |
| **bna-N_miR120** | auaaaucccaagcaucaucca | 21 | auaaauccc | 78 | -42.10 | -1.45 | 1004.58 | 880.05 | 882.96 | 828.76 | 899.51 | 928.95 | 938.00 | 6362.82 |
| **bna-N_miR121** | aacggaauauaagaacucguuucu | 24 | aacggaaua | 67 | -17.00 | -0.89 | 780.59 | 716.17 | 559.97 | 590.14 | 700.35 | 773.79 | 796.75 | 4917.75 |
| **bna-N_miR122** | uucaccggacgacuuuaaauu | 21 | uuaaggucg | 86 | -42.90 | -1.19 | 921.49 | 324.32 | 947.24 | 1307.79 | 956.54 | 1055.02 | 1012.55 | 6524.96 |
| **bna-N_miR123** | uauuuggacgacuuacauguaagu | 24 | uauuuggac | 77 | -44.30 | -1.53 | 195.31 | 299.60 | 313.81 | 314.25 | 243.25 | 403.79 | 458.32 | 2228.34 |
| **bna-N_miR124** | uaucucggacgacuuucuugu | 21 | uugaaaguc | 87 | -37.10 | -1.12 | 495.18 | 444.26 | 710.49 | 615.86 | 684.56 | 599.24 | 552.49 | 4102.07 |
| **bna-N_miR125** | uaacuaaaaaaaacuaagaacc | 22 | uaacuaaaa | 72 | -28.40 | -2.03 | 47.19 | 49.66 | 22.17 | 40.14 | 66.90 | 68.11 | 113.03 | 407.21 |
| **bna-N_miR126** | aggauuucauuuuccgucggaaug | 24 | uccgucgga | 52 | -24.70 | -0.99 | 359.09 | 672.22 | 336.54 | 265.46 | 631.04 | 603.71 | 718.27 | 3586.35 |
| **bna-N_miR127** | agcggaauauaagaacucgucucu | 24 | agcggaaua | 70 | -18.00 | -0.90 | 394.02 | 356.36 | 1013.10 | 879.33 | 1126.75 | 485.10 | 1176.37 | 5431.03 |
| **bna-N_miR128** | aacggaauauaagaacaugucucu | 24 | aacggaaua | 69 | -37.30 | -1.78 | 677.02 | 653.00 | 591.33 | 621.18 | 619.64 | 696.21 | 918.38 | 4776.76 |
| | | | | 79.9375 | -33.5344 | -1.19585 | | | | | | | | |

**Supplementary File 4:** List of all conserved miRNAs identified with precursor information and counts per million (CPM) values.

| Description | miRNA sequence | Length o | Precurso | Precurso | MFE (kc | MFEI=(1 | 0h CPM | 1h CPM | 2h CPM | 4h CPM | 8h CPM | 24h CPM | 48h CPM | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| bna-miR156d | ugacagaagagagugagcac | 20 | cgcaaagaa | 110 | -49.70 | -0.92 | 3693.70 | 3422.47 | 5030.09 | 3294.86 | 3831.63 | 2676.76 | 2628.16 | 24577.68 |
| bra-miR157a | uugacagaagauagagagcac | 21 | gugaugcug | 149 | -66.80 | -1.08 | 18780.72 | 15675.92 | 17851.70 | 14748.91 | 16001.43 | 13688.74 | 16097.46 | ####### |
| bra-miR159a | uuuggauugaagggagcucua | 21 | aaguagggc | 185 | -75.50 | -1.09 | 89017.00 | 71230.26 | 59136.43 | 48915.91 | 52439.76 | 65094.15 | 78069.35 | ####### |
| bna-miR160c | ugccuggcucccuguaugcca | 22 | gugugugcu | 115 | -40.61 | -0.83 | 3581.70 | 3362.96 | 2583.36 | 2084.88 | 3445.60 | 4508.86 | 3666.98 | 23234.34 |
| bna-miR161 | ucaaugcacugaaagugacua | 21 | uuuauugcu | 135 | -51.00 | -1.11 | 133.90 | 159.53 | 143.69 | 138.61 | 197.63 | 175.53 | 177.77 | 1126.65 |
| bna-miR162a | ucgauaaaccugugcauccag | 24 | agugaaaga | 114 | -40.40 | -0.82 | 133.90 | 159.53 | 143.69 | 138.61 | 197.63 | 175.53 | 177.77 | 1126.65 |
| bra-miR164a | uggagaagcagggcacgugca | 21 | ccuccacgu | 92 | -38.50 | -0.82 | 241.08 | 104.59 | 114.68 | 112.88 | 96.73 | 79.30 | 125.78 | 875.05 |
| bna-miR164d | uggagaagcagggcacgugcg | 21 | guaucacuu | 110 | -35.55 | -0.73 | 0.22 | 22.20 | 0.22 | 0.22 | 0.22 | 25.59 | 0.22 | 48.90 |
| bna-miR166b | ucggaccaggcuucauucccc | 21 | aaguucagg | 118 | -41.00 | -0.87 | 83411.15 | 78298.15 | ####### | 74443.58 | 91797.92 | 68442.06 | 68711.18 | ####### |
| bna-miR166f | ucggaccaggcuucaucccca | 21 | aguugaggg | 129 | -48.80 | -1.08 | 10757.91 | 11858.16 | 15085.12 | 11676.05 | 13803.66 | 9457.61 | 9763.52 | 82402.03 |
| bna-miR167d | ugaagcugccagcaugaucu | 20 | uuuugggag | 90 | -46.50 | -0.80 | 0.22 | 0.22 | 980.95 | 0.22 | 875.82 | 0.22 | 0.22 | 1857.90 |
| bna-miR168a | ucgcuuggugcaggucgggaa | 21 | uuacggcgg | 133 | -69.30 | -0.89 | 5596.44 | 2775.19 | 6322.05 | 3131.64 | 3694.77 | 4353.69 | 2582.06 | 28455.84 |
| bna-miR168b | ucgcuuggugcaggucgagaa | 21 | accgucuug | 130 | -48.10 | -0.74 | 1090.08 | 1249.92 | 1771.97 | 1305.13 | 2708.62 | 2402.99 | 1869.90 | 12398.60 |
| bna-miR169b | cagccaaggaugacuugccga | 21 | gugaccaaa | 188 | -68.50 | -1.07 | 97.77 | 64.31 | 59.80 | 96.03 | 62.52 | 100.93 | 97.34 | 578.69 |
| bna-miR169f | uagccaaggaugacuugccua | 21 | gucaaagau | 201 | -73.50 | -1.04 | 186.88 | 156.78 | 192.29 | 190.95 | 258.17 | 233.71 | 154.23 | 1373.01 |
| bna-miR169l | uagccaaggaugacuugccugc | 22 | cauggcgaa | 199 | -82.30 | -1.07 | 361.50 | 333.48 | 364.76 | 291.19 | 359.94 | 434.38 | 352.38 | 2497.63 |
| bna-miR169m | ugagccaaagaugacuugccg | 21 | uauauguau | 163 | -68.50 | -1.16 | 106.20 | 97.27 | 146.04 | 158.12 | 193.24 | 62.14 | 72.81 | 835.83 |
| bna-miR169n | cagccaaggaugacuugccgg | 21 | agaauugca | 150 | -72.60 | -1.13 | 191.70 | 183.33 | 203.27 | 229.09 | 256.41 | 160.61 | 191.51 | 1415.92 |
| bna-miR171a | uugagccgugccaauaucacg | 21 | uggucaagc | 104 | -39.70 | -1.10 | 31.53 | 24.03 | 58.24 | 2.88 | 22.16 | 12.16 | 61.04 | 212.04 |
| bna-miR171b | uugagccgugccaauaucacg | 21 | gguaacgcg | 101 | -43.27 | -0.95 | 12.27 | 19.45 | 11.98 | 0.22 | 10.75 | 30.81 | 0.22 | 85.71 |
| bra-miR171c | uugagccgugccaauaucacg | 21 | gcgagauau | 88 | -36.60 | -0.96 | 12.27 | 19.45 | 11.98 | 0.22 | 10.75 | 30.81 | 0.22 | 85.71 |
| bna-miR171d | uugagccgugccaauaucacg | 21 | acaaugcga | 96 | -42.70 | -1.04 | 109.81 | 116.50 | 101.35 | 80.95 | 128.32 | 90.49 | 72.81 | 700.23 |
| bna-miR171f | ugauugagccgcgccaauauc | 21 | acgaaagag | 119 | -40.30 | -0.78 | 418.10 | 290.45 | 304.40 | 222.88 | 295.02 | 273.25 | 254.29 | 2058.38 |
| bna-miR172b | ggaaucuugaugaugcugcau | 21 | uaguugcag | 95 | -56.21 | -1.37 | 77.30 | 48.75 | 65.29 | 49.90 | 90.59 | 93.47 | 136.57 | 561.87 |
| bra-miR172b-5p | gcagcaccauuaagauucaca | 21 | uguaggugc | 107 | -46.50 | -1.29 | 548.16 | 496.44 | 430.62 | 297.40 | 521.37 | 544.03 | 759.47 | 3597.50 |
| bna-miR172c | ggaaucuugaugaugcugcau | 21 | cagccggua | 138 | -49.06 | -1.07 | 5.04 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 6.38 |
| bna-miR390a | aagcucaggagggauagcgcc | 21 | auuucaggu | 144 | -63.00 | -1.07 | 440.98 | 391.15 | 461.19 | 376.35 | 342.39 | 318.75 | 341.59 | 2672.41 |
| bna-miR393 | uccaaagggaucgcauugauc | 21 | uccaaaggg | 127 | -36.80 | -0.86 | 350.66 | 356.36 | 309.89 | 263.69 | 404.68 | 439.60 | 437.72 | 2562.61 |
| bna-miR394a | uuggcauucuguccaccucc | 20 | acagaguuc | 116 | -48.60 | -1.01 | 192.91 | 0.22 | 95.08 | 289.41 | 0.22 | 331.43 | 293.52 | 1202.81 |
| bna-miR394b | uuggcauucuguccaccucc | 20 | uuacagaga | 112 | -48.00 | -0.94 | 715.56 | 891.95 | 549.78 | 26.84 | 764.40 | 197.16 | 241.53 | 3387.21 |
| bna-miR395c | cugaaguguuuggggggaacuc | 21 | uguuuccua | 98 | -43.88 | -1.16 | 5.04 | 6.63 | 5.71 | 8.21 | 9.00 | 2.46 | 7.09 | 44.14 |
| bna-miR395d | cugaaguguuuggggggacuc | 21 | gccccaug | 117 | -38.55 | -0.92 | 0.22 | 0.22 | 0.22 | 3.77 | 0.22 | 0.22 | 0.22 | 5.11 |
| bna-miR395e | cugaaguguuuggggggacuc | 21 | ccccuugag | 96 | -51.35 | -1.20 | 0.22 | 2.05 | 4.93 | 6.43 | 1.98 | 6.94 | 1.20 | 23.76 |
| bna-miR395f | cugaaguguuuggggggacuc | 21 | guccucuug | 108 | -49.30 | -1.20 | 14.67 | 5.72 | 0.22 | 0.22 | 5.49 | 0.22 | 1.20 | 27.75 |
| bna-miR397a | ucauugagugcagcguugaugu | 22 | gaacaucau | 96 | -38.31 | -1.20 | 41.17 | 82.62 | 95.08 | 96.03 | 110.77 | 156.13 | 115.97 | 697.78 |
| bna-miR397b | ucauugagugcagcguugaugu | 22 | gaacaucau | 96 | -38.20 | -1.23 | 41.17 | 76.21 | 91.16 | 91.59 | 106.38 | 131.51 | 102.24 | 640.28 |
| bol-miR398a-5p | gagugucaugagaacacggaucc | 24 | ucucaaagg | 95 | -33.80 | -0.87 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 8.43 | 0.22 | 9.77 |
| bol-miR398a-p | uguguucucaggucaccccuu | 21 | ucucaaagg | 95 | -33.80 | -0.87 | 0.22 | 114.63 | 0.22 | 378.09 | 560.86 | 482.77 | 0.22 | 1537.02 |
| bna-miR403 | uuagauucacgcacaaacucg | 21 | agagaagag | 111 | -41.00 | -1.05 | 14647.68 | 13180.19 | 13478.01 | 13839.65 | 14441.49 | 12435.51 | 13954.10 | 95976.64 |
| bra-miR824 | uagaccauuugugagaaggga | 21 | ccucgaguu | 632 | -184.90 | -0.77 | 2036.63 | 1555.71 | 1209.08 | 1198.68 | 1291.69 | 1220.63 | 1511.85 | 10024.28 |
| bna-miR860 | ucaauacauuggacuacauau | 21 | uggucaagu | 115 | -63.00 | -1.58 | 13.47 | 9.38 | 18.25 | 11.76 | 2.86 | 8.43 | 11.01 | 75.16 |
| bna-miR1140 | acagccuaaaccaaucggagc | 21 | cucaaucuu | 150 | -66.70 | -1.19 | 4300.65 | 4378.29 | 4650.66 | 4207.67 | 4241.36 | 3138.51 | 3290.30 | 28207.43 |
| bra-miR1885b | uacaucuucucgcggaagcuc | 22 | uugucucac | 355 | -160.60 | -1.22 | 5134.00 | 3290.64 | 3243.45 | 2657.05 | 3270.13 | 4138.11 | 4608.68 | 26342.05 |
| bna-miR2111b | uaaucugcauccugagguuua | 21 | gcacuugau | 119 | -43.78 | -1.18 | 0.22 | 4.80 | 0.22 | 0.22 | 0.22 | 3.95 | 0.22 | 9.87 |
| bna-miR2111c | uaaucugcauccuggguuua | 21 | uauugguga | 136 | -51.99 | -1.22 | 1.43 | 0.22 | 1.01 | 1.11 | 1.10 | 0.22 | 0.22 | 5.32 |
| bra-miR5711 | uguuuuuggguuucuaccga | 21 | auugauuac | 108 | -40.70 | -1.04 | 48.39 | 31.35 | 46.48 | 34.82 | 40.58 | 21.86 | 34.56 | 258.04 |
| bra-miR5712 | aauauuaauauaauauggugag | 21 | guucacauu | 142 | -53.10 | -1.44 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 10.03 | 11.37 |
| bra-miR5713 | aggcuuagaagaacguuuguu | 21 | auaagcaga | 446 | -52.76 | -1.25 | 1.43 | 0.22 | 0.22 | 0.22 | 1.10 | 0.22 | 1.20 | 4.63 |
| bra-miR5714 | agacucuacgacaucaagaaac | 22 | gguaugagc | 236 | -35.85 | -0.92 | 7.45 | 7.55 | 3.36 | 8.21 | 7.24 | 0.97 | 3.17 | 37.94 |
| bra-miR5715 | acgugauaagccucugaagaa | 21 | guauuagau | 186 | -36.99 | -0.86 | 0.22 | 0.22 | 1.01 | 0.22 | 0.22 | 0.22 | 0.22 | 2.35 |
| bra-miR5716 | uuggauaauugaagauauaaa | 21 | gauccagac | 206 | -109.40 | -1.56 | 8.65 | 2.97 | 6.50 | 0.22 | 9.87 | 0.22 | 2.19 | 30.63 |
| bra-miR5717 | guuuggauuguuugccuuggc | 21 | ucucucucu | 112 | -61.20 | -1.22 | 0.22 | 24.03 | 10.42 | 10.87 | 17.77 | 15.14 | 16.90 | 95.35 |
| bra-miR5718 | ucagaaccaaacacagaacaag | 22 | ugauuuaug | 234 | -127.90 | -1.44 | 2441.27 | 2519.76 | 2744.86 | 2453.90 | 2741.96 | 2248.57 | 2325.05 | 17475.37 |
| bra-miR5719 | uugugaugauaauacgacuuc | 21 | ugaucucuc | 204 | -81.60 | -1.18 | 2308.80 | 2309.19 | 2065.95 | 1839.15 | 2283.10 | 2229.18 | 2379.00 | 15414.37 |
| bra-miR5720 | uugugauuugguuggaauauc | 21 | aaacuugua | 290 | -58.86 | -2.19 | 133.90 | 148.54 | 127.22 | 118.21 | 151.13 | 124.80 | 140.50 | 944.29 |
| bra-miR5721 | aaaaauggagugagaaaugga | 21 | gggcaucuu | 178 | -42.87 | -1.34 | 23.10 | 5.72 | 6.50 | 5.55 | 4.61 | 12.16 | 15.92 | 73.55 |
| bra-miR5722 | ugaaauagagucauguggaacg | 22 | uuacaguca | 216 | -76.30 | -1.34 | 0.22 | 0.22 | 0.22 | 0.22 | 1.98 | 0.22 | 0.22 | 3.32 |
| bra-miR5723 | aaugugcugcaauaucucugc | 21 | gcuauggac | 163 | -53.74 | -1.44 | 36.35 | 21.28 | 33.93 | 28.61 | 31.81 | 21.86 | 36.52 | 210.36 |
| bra-miR5724 | aaccgccgguuugauaauagc | 21 | cagauuguc | 310 | -42.61 | -1.02 | 32.74 | 34.10 | 30.01 | 27.72 | 33.56 | 20.36 | 21.80 | 200.31 |
| bra-miR5725 | auuuggcacaaucugaucugc | 21 | aagguaucu | 205 | -55.66 | -1.84 | 124.26 | 126.57 | 137.42 | 112.88 | 151.13 | 123.31 | 142.46 | 918.03 |
| bra-miR5726 | caaagguugcuugaauaaggu | 21 | agaugaagg | 342 | -63.83 | -1.54 | 141.12 | 126.57 | 160.15 | 115.54 | 132.70 | 130.77 | 128.73 | 935.58 |
| bra-miR5654a | auaaaucccaagcaucaucca | 21 | uaaugaugu | 195 | -69.00 | -0.93 | 1004.58 | 880.05 | 882.96 | 828.76 | 899.51 | 928.95 | 938.00 | 6362.82 |
| bna-miR6028 | uggagaguaaggacauucaga | 21 | acaagcacg | 131 | -48.50 | -0.87 | 581.88 | 467.14 | 350.65 | 300.06 | 359.06 | 528.37 | 618.22 | 3205.39 |
| bna-miR6029 | ugggguugugauuucaggcuu | 21 | aagagauac | 116 | -62.50 | -1.45 | 0.22 | 0.22 | 0.22 | 0.22 | 9.00 | 8.43 | 14.94 | 33.26 |
| bna-miR6030 | uccacccauaccauacagaccc | 22 | ucagggauu | 113 | -55.30 | -1.06 | 312.13 | 201.64 | 216.60 | 182.08 | 195.87 | 343.37 | 292.54 | 1744.23 |
| bna-miR6031 | aagagguucggagcgguuugaag | 24 | aaacugcaa | 91 | -40.22 | -1.14 | 156.78 | 149.45 | 186.02 | 132.40 | 167.80 | 133.01 | 159.14 | 1084.59 |
| bna-miR6032 | uggagcaucaacagaucucgg | 21 | uuauuuugu | 100 | -38.80 | -0.99 | 1.43 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 0.22 | 2.77 |
| bna-miR6034 | ucugauguauauagcuuuggg | 21 | auauacucu | 127 | -62.52 | -1.76 | 59.23 | 72.55 | 54.32 | 48.13 | 66.90 | 55.43 | 68.89 | 425.44 |
| bna-miR6035 | uggaguagaaaaugcagucgu | 21 | aaacugcaa | 91 | -36.60 | -1.14 | 18.29 | 13.04 | 2.58 | 7.32 | 9.00 | 4.70 | 9.05 | 63.97 |
| bna-miR6036 | auaguacuaguacuugcaugauc | 24 | ucaguuagc | 155 | -20.58 | -0.63 | 1.43 | 0.22 | 0.22 | 0.22 | 1.98 | 0.22 | 2.19 | 6.49 |
| | | | | 156.7714 | -56.2062 | -1.11676 | | | | | | | | |

**Supplementary File 5:** List of novel DE miRNAs identified in this study.

| | logFC 1h/0h | logFC 2h/0h | logFC 4h/0h | logFC 8h/0h | logFC 24h/0h | logFC 48h/0h |
|---|---|---|---|---|---|---|
| **bna-N_miR1** | 0.13 | 0.60 | -6.61 | -6.61 | -6.61 | -6.61 |
| **bna-N_miR2** | 0.01 | -0.44 | 0.58 | -8.64 | -8.64 | -8.64 |
| **bna-N_miR3** | 0.23 | -0.45 | 0.73 | -8.02 | -8.02 | -8.02 |
| **bna-N_miR4** | 6.22 | 6.15 | 6.18 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR5** | 6.85 | 7.62 | 6.70 | 6.31 | 0.00 | 0.00 |
| **bna-N_miR6** | 1.16 | 0.94 | 1.11 | -1.52 | 0.64 | 1.15 |
| **bna-N_miR7** | -0.32 | -0.34 | -1.94 | -1.14 | 0.37 | -0.26 |
| **bna-N_miR8** | 0.15 | -1.14 | -4.31 | -0.44 | 0.43 | 1.15 |
| **bna-N_miR9** | 1.04 | 1.52 | 1.28 | 1.31 | 2.06 | 2.18 |
| **bna-N_miR10** | 0.10 | 1.28 | 0.84 | 1.38 | -0.28 | 0.53 |
| **bna-N_miR11** | 7.36 | 6.63 | 8.04 | 7.23 | 6.92 | 7.66 |
| **bna-N_miR12** | 7.85 | 7.62 | 7.25 | 7.50 | 8.07 | 8.39 |
| **bna-N_miR13** | 9.17 | 8.88 | 10.10 | 9.28 | 8.36 | 10.41 |
| **bna-N_miR14** | 7.85 | 7.21 | 7.46 | 6.89 | 7.45 | 0.00 |
| **bna-N_miR15** | -9.26 | -9.26 | 0.34 | 0.05 | 0.11 | 1.10 |
| **bna-N_miR16** | -9.31 | -9.31 | -9.31 | 0.14 | -0.33 | 0.33 |
| **bna-N_miR17** | 0.00 | 0.00 | 0.00 | 0.00 | 6.45 | 6.61 |
| **bna-N_miR18** | 0.00 | 0.00 | 0.00 | 0.00 | 6.45 | 7.31 |
| **bna-N_miR19** | -6.90 | 1.93 | 1.05 | 1.50 | 0.93 | 1.19 |
| **bna-N_miR20** | 0.60 | -7.68 | 0.67 | 1.31 | 0.38 | 1.66 |
| **bna-N_miR21** | -0.92 | -9.91 | -9.91 | 0.11 | -0.25 | 0.47 |
| **bna-N_miR22** | -0.07 | 1.17 | -14.78 | 0.59 | -0.43 | 0.71 |
| **bna-N_miR23** | 0.00 | 0.00 | 7.46 | 7.07 | 7.27 | 0.00 |
| **bna-N_miR24** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR25** | 6.37 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR26** | 6.37 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR27** | 6.63 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR28** | 8.71 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR29** | 9.23 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR30** | 6.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR31** | 6.37 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR32** | 5.65 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR33** | 5.87 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR34** | 6.22 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR35** | 6.63 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR36** | 6.22 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR37** | 7.50 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR38** | 7.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR39** | 7.95 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR40** | 6.63 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |

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|---|---|---|---|---|---|---|
| **bna-N_miR41** | 6.95 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR42** | 6.51 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR43** | 0.00 | 6.99 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR44** | 0.00 | 8.06 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR45** | 0.00 | 8.65 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR46** | 0.00 | 7.40 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR47** | 0.00 | 7.21 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR48** | 0.00 | 6.73 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR49** | 0.00 | 6.15 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR50** | 0.00 | 7.07 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR51** | 0.00 | 7.44 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR52** | 0.00 | 4.06 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR53** | 0.00 | 6.53 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR54** | 0.00 | 9.34 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR55** | 0.00 | 7.52 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR56** | 0.00 | 7.04 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR57** | 0.00 | 6.99 | 0.00 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR58** | 0.00 | 0.00 | 9.53 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR59** | 0.00 | 0.00 | 6.70 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR60** | 0.00 | 0.00 | 6.18 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR61** | 0.00 | 0.00 | 7.80 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR62** | 0.00 | 0.00 | 6.33 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR63** | 0.00 | 0.00 | 6.59 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR64** | 0.00 | 0.00 | 6.33 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR65** | 0.00 | 0.00 | 6.33 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR66** | 0.00 | 0.00 | 7.64 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR67** | 0.00 | 0.00 | 7.90 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR68** | 0.00 | 0.00 | 5.82 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR69** | 0.00 | 0.00 | 6.33 | 0.00 | 0.00 | 0.00 |
| **bna-N_miR70** | 0.00 | 0.00 | 0.00 | 8.11 | 0.00 | 0.00 |
| **bna-N_miR71** | 0.00 | 0.00 | 0.00 | 6.31 | 0.00 | 0.00 |
| **bna-N_miR72** | 0.00 | 0.00 | 0.00 | 5.81 | 0.00 | 0.00 |
| **bna-N_miR73** | 0.00 | 0.00 | 0.00 | 6.45 | 0.00 | 0.00 |
| **bna-N_miR74** | 0.00 | 0.00 | 0.00 | 5.99 | 0.00 | 0.00 |
| **bna-N_miR75** | 0.00 | 0.00 | 0.00 | 6.79 | 0.00 | 0.00 |
| **bna-N_miR76** | 0.00 | 0.00 | 0.00 | 5.81 | 0.00 | 0.00 |
| **bna-N_miR77** | 0.00 | 0.00 | 0.00 | 6.79 | 0.00 | 0.00 |
| **bna-N_miR78** | 0.00 | 0.00 | 0.00 | 5.81 | 0.00 | 0.00 |
| **bna-N_miR79** | 0.00 | 0.00 | 0.00 | 0.00 | 6.34 | 0.00 |
| **bna-N_miR80** | 0.00 | 0.00 | 0.00 | 0.00 | 6.34 | 0.00 |
| **bna-N_miR81** | 0.00 | 0.00 | 0.00 | 0.00 | 7.39 | 0.00 |
| **bna-N_miR82** | 0.00 | 0.00 | 0.00 | 0.00 | 6.08 | 0.00 |
| **bna-N_miR83** | 0.00 | 0.00 | 0.00 | 0.00 | 6.34 | 0.00 |
| **bna-N_miR84** | 0.00 | 0.00 | 0.00 | 0.00 | 7.00 | 0.00 |
| **bna-N_miR85** | 0.00 | 0.00 | 0.00 | 0.00 | 7.07 | 0.00 |
| **bna-N_miR86** | 0.00 | 0.00 | 0.00 | 0.00 | 7.95 | 0.00 |
| **bna-N_miR87** | 0.00 | 0.00 | 0.00 | 0.00 | 5.58 | 0.00 |
| **bna-N_miR88** | 0.00 | 0.00 | 0.00 | 0.00 | 5.58 | 0.00 |
| **bna-N_miR89** | 0.00 | 0.00 | 0.00 | 0.00 | 6.08 | 0.00 |
| **bna-N_miR90** | 0.00 | 0.00 | 0.00 | 0.00 | 8.97 | 0.00 |

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|---|---|---|---|---|---|---|
| **bna-N_miR84** | 0.00 | 0.00 | 0.00 | 0.00 | 7.00 | 0.00 |
| **bna-N_miR85** | 0.00 | 0.00 | 0.00 | 0.00 | 7.07 | 0.00 |
| **bna-N_miR86** | 0.00 | 0.00 | 0.00 | 0.00 | 7.95 | 0.00 |
| **bna-N_miR87** | 0.00 | 0.00 | 0.00 | 0.00 | 5.58 | 0.00 |
| **bna-N_miR88** | 0.00 | 0.00 | 0.00 | 0.00 | 5.58 | 0.00 |
| **bna-N_miR89** | 0.00 | 0.00 | 0.00 | 0.00 | 6.08 | 0.00 |
| **bna-N_miR90** | 0.00 | 0.00 | 0.00 | 0.00 | 8.97 | 0.00 |
| **bna-N_miR91** | 0.00 | 0.00 | 0.00 | 0.00 | 6.34 | 0.00 |
| **bna-N_miR92** | 0.00 | 0.00 | 0.00 | 0.00 | 6.75 | 0.00 |
| **bna-N_miR93** | 0.00 | 0.00 | 0.00 | 0.00 | 7.39 | 0.00 |
| **bna-N_miR94** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 8.35 |
| **bna-N_miR95** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 5.75 |
| **bna-N_miR96** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.95 |
| **bna-N_miR97** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.95 |
| **bna-N_miR98** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 5.75 |
| **bna-N_miR99** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 8.49 |
| **bna-N_miR100** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.32 |
| **bna-N_miR101** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 5.75 |
| **bna-N_miR102** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.32 |
| **bna-N_miR103** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 7.05 |
| **bna-N_miR104** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.47 |

**Supplementary File 6**: List of known DE miRNAs identified in this study.

| Description | logFC 1h/0h | logFC 2h/0h | logFC 4h/0h | logFC 8h/0h | logFC 24h/0h | logFC 48h/0h |
|---|---|---|---|---|---|---|
| miR162a | -0.23 | 0.29 | 0.04 | -0.38 | 0.00 | -1.86 |
| miR164a | -1.21 | -1.07 | -1.10 | -1.32 | -1.61 | -0.94 |
| miR164d | 7.63 | 0.00 | 0.00 | 0.00 | 7.83 | 0.00 |
| miR166b | -0.09 | 0.31 | -0.16 | 0.14 | -0.29 | -0.28 |
| miR166f | 0.14 | 0.49 | 0.12 | 0.36 | -0.19 | -0.14 |
| miR167d | 0.00 | 13.10 | 0.00 | 12.94 | 0.00 | 0.00 |
| miR168a | -1.01 | 0.18 | -0.84 | -0.60 | -0.36 | -1.12 |
| miR168b | 0.20 | 0.70 | 0.26 | 1.31 | 1.14 | 0.78 |
| miR171a | -0.39 | 0.89 | -3.50 | -0.51 | -1.38 | 0.96 |
| miR171b | 0.67 | -0.03 | -6.76 | -0.19 | 1.34 | -6.76 |
| miR393 | 0.02 | -1.18 | -1.41 | 0.21 | 0.33 | 0.32 |
| miR394a | -10.75 | -1.02 | 0.59 | -10.75 | 0.78 | 0.61 |
| miR394b | 0.32 | -0.38 | -4.74 | 0.10 | -1.86 | -1.57 |
| miR395c | 0.18 | 0.85 | 0.50 | 0.72 | -1.07 | 4.00 |
| miR395e | 4.12 | 5.43 | 5.82 | 4.06 | 5.93 | 3.29 |
| miR395f | -1.38 | -7.03 | -7.03 | -1.44 | -7.03 | -3.74 |
| miR397a | 1.01 | 1.21 | 1.22 | 1.43 | 1.93 | 1.50 |
| miR397b | 0.89 | 1.15 | 1.16 | 1.37 | 1.68 | 1.31 |
| miR398a-5p | 0.00 | 0.00 | 0.00 | 0.00 | 6.22 | 0.00 |
| miR398a-3p | -10.00 | 1.72 | 2.29 | 2.08 | -10.00 | 0.95 |
| miR5712 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 6.47 |
| miR5716 | -1.58 | -0.42 | -6.26 | 0.19 | -6.26 | -2.04 |
| miR5717 | 7.74 | 6.53 | 6.59 | 7.30 | 7.07 | 7.23 |
| miR6029 | -9.02 | -0.07 | -9.02 | -0.88 | -3.41 | -9.02 |
| miR6035 | -0.49 | -2.88 | -1.33 | -1.03 | -1.99 | -1.02 |

**Supplementary File 7**: Predicted targets of conserved miRNAs by psRNATarget.

| miRNA_Name | Target_Desc. |
|---|---|
| miR164a | homologue to UniRef100_Q9FRV4 Cluster: Protein CUP-SHAPED COTYLEDON 1; n=1; Arabidopsis thaliana\|Rep: Protein CUP-SHAPED COTYLEDON 1 - Arabidopsis thaliana (Mouse-ear cress), partial (71%) |
| miR164a | homologue to UniRef100_Q9FLJ2 Cluster: NAM (No apical meristem)-like protein; n=2; Arabidopsis thaliana\|Rep: NAM (No apical meristem)-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (90%) |
| miR164a | similar to UniRef100_Q84TE6 Cluster: NAC domain-containing protein 21/22; n=2; Arabidopsis thaliana\|Rep: NAC domain-containing protein 21/22 - Arabidopsis thaliana (Mouse-ear cress), partial (93%) |
| miR164a | homologue to UniRef100_P94014 Cluster: Germin-like protein subfamily 2 member 1 precursor; n=1; Arabidopsis thaliana\|Rep: Germin-like protein subfamily 2 member 1 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (83%) |
| miR164a | homologue to UniRef100_Q3E902 Cluster: 40S ribosomal protein S21-2; n=2; Arabidopsis thaliana\|Rep: 40S ribosomal protein S21-2 - Arabidopsis thaliana (Mouse-ear cress), complete |
| miR164d | similar to UniRef100_Q9FRV4 Cluster: Protein CUP-SHAPED COTYLEDON 1; n=1; Arabidopsis thaliana\|Rep: Protein CUP-SHAPED COTYLEDON 1 - Arabidopsis thaliana (Mouse-ear cress), partial (75%) |
| miR164d | homologue to UniRef100_Q9FLJ2 Cluster: NAM (No apical meristem)-like protein; n=2; Arabidopsis thaliana\|Rep: NAM (No apical meristem)-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (90%) |
| miR164d | similar to UniRef100_Q84TE6 Cluster: NAC domain-containing protein 21/22; n=2; Arabidopsis thaliana\|Rep: NAC domain-containing protein 21/22 - Arabidopsis thaliana (Mouse-ear cress), partial (93%) |
| miR164d | homologue to UniRef100_P94014 Cluster: Germin-like protein subfamily 2 member 1 precursor; n=1; Arabidopsis thaliana\|Rep: Germin-like protein subfamily 2 member 1 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (83%) |
| miR166b | UniRef100_Q9SE43 Cluster: Homeodomain-leucine zipper protein interfascicular fiberless 1; n=2; Arabidopsis thaliana\|Rep: Homeodomain-leucine zipper protein interfascicular fiberless 1 - Arabidopsis thaliana (Mouse-ear cress), partial (25%) |
| miR166b | homologue to UniRef100_O04292 Cluster: HD-Zip protein; n=3; Arabidopsis thaliana\|Rep: HD-Zip protein - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| miR166b | similar to UniRef100_Q9LMV6 Cluster: F5M15.19; n=1; Arabidopsis thaliana\|Rep: F5M15.19 - Arabidopsis thaliana (Mouse-ear cress), partial (83%) |
| miR166f | UniRef100_Q9SE43 Cluster: Homeodomain-leucine zipper protein interfascicular fiberless 1; n=2; Arabidopsis thaliana\|Rep: Homeodomain-leucine zipper protein interfascicular fiberless 1 - Arabidopsis thaliana (Mouse-ear cress), partial (25%) |
| miR166f | homologue to UniRef100_O04292 Cluster: HD-Zip protein; n=3; Arabidopsis thaliana\|Rep: HD-Zip protein - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| miR166f | UniRef100_Q08700 Cluster: S-locus glycoprotein type II precursor; n=2; Brassica\|Rep: S-locus glycoprotein type II precursor - Brassica napus (Rape), complete |
| miR166f | homologue to UniRef100_Q9SP02 Cluster: Peptidyl-prolyl cis-trans isomerase CYP20-1 precursor; n=1; Arabidopsis thaliana\|Rep: Peptidyl-prolyl cis-trans isomerase CYP20-1 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| miR167d | homologue to UniRef100_Q9SRX3 Cluster: Endoglucanase 1 precursor; n=1; Arabidopsis thaliana\|Rep: Endoglucanase 1 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (95%) |
| miR168a | UniRef100_Q2A977 Cluster: RNA recognition motif (RRM)-containing protein; n=1; Brassica oleracea\|Rep: RNA recognition motif (RRM)-containing protein - Brassica oleracea (Wild cabbage), complete |
| miR168b | UniRef100_Q2A977 Cluster: RNA recognition motif (RRM)-containing protein; n=1; Brassica oleracea\|Rep: RNA recognition motif (RRM)-containing protein - Brassica oleracea (Wild cabbage), complete |
| miR171a | similar to UniRef100_Q8GX15 Cluster: Ap2 SCARECROW-like protein; n=1; Arabidopsis thaliana\|Rep: Ap2 SCARECROW-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (81%) |
| miR171a | similar to UniRef100_O81316 Cluster: F6N15.20 protein; n=2; Arabidopsis thaliana\|Rep: F6N15.20 protein - Arabidopsis thaliana (Mouse-ear cress), partial (58%) |
| miR171b | similar to UniRef100_Q8GX15 Cluster: Ap2 SCARECROW-like protein; n=1; Arabidopsis thaliana\|Rep: Ap2 SCARECROW-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (81%) |
| miR171b | similar to UniRef100_O81316 Cluster: F6N15.20 protein; n=2; Arabidopsis thaliana\|Rep: F6N15.20 protein - Arabidopsis thaliana (Mouse-ear cress), partial (58%) |
| miR393 | similar to UniRef100_Q9LPW7 Cluster: Protein AUXIN SIGNALING F-BOX 3; n=1; Arabidopsis thaliana\|Rep: Protein AUXIN SIGNALING F-BOX 3 - Arabidopsis thaliana (Mouse-ear cress), partial (64%) |
| miR393 | homologue to UniRef100_Q570C0 Cluster: Protein TRANSPORT INHIBITOR RESPONSE 1; n=2; Arabidopsis thaliana\|Rep: Protein TRANSPORT INHIBITOR RESPONSE 1 - Arabidopsis thaliana (Mouse-ear cress), partial (55%) |
| miR393 | homologue to UniRef100_Q9LK48 Cluster: Similarity to DNA-binding protein; n=1; Arabidopsis thaliana\|Rep: Similarity to DNA-binding protein - Arabidopsis thaliana (Mouse-ear cress), partial (85%) |
| miR393 | similar to UniRef100_Q9ZR12 Cluster: GRR1-like protein 1; n=1; Arabidopsis thaliana\|Rep: GRR1-like protein 1 - Arabidopsis thaliana (Mouse-ear cress), partial (67%) |
| miR394a | homologue to UniRef100_Q9FZK1 Cluster: F-box only protein 6; n=1; Arabidopsis thaliana\|Rep: F-box only protein 6 - Arabidopsis thaliana (Mouse-ear cress), partial (40%) |
| miR394b | homologue to UniRef100_Q9FZK1 Cluster: F-box only protein 6; n=1; Arabidopsis thaliana\|Rep: F-box only protein 6 - Arabidopsis thaliana (Mouse-ear cress), partial (40%) |
| miR395c | homologue to UniRef100_Q9ZNZ9 Cluster: ATP sulfurylase precursor; n=1; Brassica juncea\|Rep: ATP sulfurylase precursor - Brassica juncea (Leaf mustard) (Indian mustard), complete |
| miR395c | UniRef100_Q6ZZ95 Cluster: Plasma membrane sulphate transporter; n=1; Brassica oleracea var. acephala\|Rep: Plasma membrane sulphate transporter - Brassica oleracea var. acephala (Flowering kale), partial (25%) |
| miR395c | similar to UniRef100_Q9LS03 Cluster: Allene oxide cyclase 1, chloroplast precursor; n=2; Arabidopsis thaliana\|Rep: Allene oxide cyclase 1, chloroplast precursor - Arabidopsis thaliana (Mouse-ear cress), partial (95%) |
| miR395e | homologue to UniRef100_Q9ZNZ9 Cluster: ATP sulfurylase precursor; n=1; Brassica juncea\|Rep: ATP sulfurylase precursor - Brassica juncea (Leaf mustard) (Indian mustard), complete |
| miR395e | homologue to UniRef100_Q1H595 Cluster: At1g03330; n=1; Arabidopsis thaliana\|Rep: At1g03330 - Arabidopsis thaliana (Mouse-ear cress), complete |
| miR395f | homologue to UniRef100_Q9ZNZ9 Cluster: ATP sulfurylase precursor; n=1; Brassica juncea\|Rep: ATP sulfurylase precursor - Brassica juncea (Leaf mustard) (Indian mustard), complete |
| miR395f | homologue to UniRef100_Q1H595 Cluster: At1g03330; n=1; Arabidopsis thaliana\|Rep: At1g03330 - Arabidopsis thaliana (Mouse-ear cress), complete |
| miR395f | similar to UniRef100_Q2A9R4 Cluster: Ethylene responsive element binding factor-related; n=1; Brassica oleracea\|Rep: Ethylene responsive element binding factor-related - Brassica oleracea (Wild cabbage), partial (7%) |
| miR397a | homologue to UniRef100_O80434 Cluster: Laccase-4 precursor; n=1; Arabidopsis thaliana\|Rep: Laccase-4 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (71%) |
| miR397a | homologue to UniRef100_Q9SYP2 Cluster: Pyrophosphate-dependent phosphofructokinase alpha subunit; n=1; Arabidopsis thaliana\|Rep: Pyrophosphate-dependent phosphofructokinase alpha subunit - Arabidopsis thaliana (Mouse-ear cress), partial (48%) |
| miR397a | homologue to UniRef100_Q8H1P6 Cluster: Aminopeptidase P; n=2; Arabidopsis thaliana\|Rep: Aminopeptidase P - Arabidopsis thaliana (Mouse-ear cress), partial (84%) |
| miR397b | homologue to UniRef100_O80434 Cluster: Laccase-4 precursor; n=1; Arabidopsis thaliana\|Rep: Laccase-4 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (71%) |
| miR397b | similar to UniRef100_Q8VY97 Cluster: UPF0480 protein At4g32130 precursor; n=1; Arabidopsis thaliana\|Rep: UPF0480 protein At4g32130 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| miR397b | homologue to UniRef100_Q9SYP2 Cluster: Pyrophosphate-dependent phosphofructokinase alpha subunit; n=1; Arabidopsis thaliana\|Rep: Pyrophosphate-dependent phosphofructokinase alpha subunit - Arabidopsis thaliana (Mouse-ear cress), partial (48%) |
| miR398a-3p | homologue to UniRef100_P51420 Cluster: 60S ribosomal protein L31-3; n=1; Arabidopsis thaliana\|Rep: 60S ribosomal protein L31-3 - Arabidopsis thaliana (Mouse-ear cress), complete |
| miR398a-3p | homologue to UniRef100_Q9FJE8 Cluster: Probable histone H2A.7; n=1; Arabidopsis thaliana\|Rep: Probable histone H2A.7 - Arabidopsis thaliana (Mouse-ear cress), partial (86%) |
| miR5712 | similar to UniRef100_Q49KZ4 Cluster: NADH-plastoquinone oxidoreductase subunit K; n=1; Eucalyptus globulus subsp. globulus\|Rep: NADH-plastoquinone oxidoreductase subunit K - Eucalyptus globulus subsp. globulus (Tasmanian blue gum), partial (97%) |
| miR5712 | similar to UniRef100_P46604 Cluster: Homeobox-leucine zipper protein HAT22; n=1; Arabidopsis thaliana\|Rep: Homeobox-leucine zipper protein HAT22 - Arabidopsis thaliana (Mouse-ear cress), partial (97%) |
| miR5716 | homologue to UniRef100_Q9SLN5 Cluster: Methionine aminopeptidase 1A; n=3; Arabidopsis thaliana\|Rep: Methionine aminopeptidase 1A - Arabidopsis thaliana (Mouse-ear cress), complete |
| miR5716 | similar to UniRef100_Q2A986 Cluster: SLL3 ORF2 protein; n=1; Brassica oleracea\|Rep: SLL3 ORF2 protein - Brassica oleracea (Wild cabbage), partial (64%) |
| miR5716 | similar to UniRef100_Q0D6L9 Cluster: Os07g0467200 protein; n=1; Oryza sativa Japonica Group\|Rep: Os07g0467200 protein - Oryza sativa subsp. japonica (Rice), partial (65%) |
| miR5717 | homologue to UniRef100_A7J146 Cluster: C2H2 zinc finger protein; n=1; Brassica carinata\|Rep: C2H2 zinc finger protein - Brassica carinata (Ethiopian mustard) (Abyssinian cabbage), partial (94%) |
| miR5717 | similar to UniRef100_Q8LBH4 Cluster: Ids4-like protein; n=1; Arabidopsis thaliana\|Rep: Ids4-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (81%) |
| miR5717 | similar to UniRef100_Q9LIM2 Cluster: Similarity to 26S proteasome subunit 4; n=1; Arabidopsis thaliana\|Rep: Similarity to 26S proteasome subunit 4 - Arabidopsis thaliana (Mouse-ear cress), partial (27%) |
| miR5717 | homologue to UniRef100_Q8GW46 Cluster: Axial regulator YABBY 5; n=1; Arabidopsis thaliana\|Rep: Axial regulator YABBY 5 - Arabidopsis thaliana (Mouse-ear cress), complete |
| miR5717 | similar to UniRef100_Q9LTH9 Cluster: Zinc transporter 2 precursor; n=2; Arabidopsis thaliana\|Rep: Zinc transporter 2 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (95%) |
| miR5717 | similar to UniRef100_Q9LXA7 Cluster: Bromodomain protein-like; n=1; Arabidopsis thaliana\|Rep: Bromodomain protein-like - Arabidopsis thaliana (Mouse-ear cress), partial (48%) |
| miR5717 | similar to UniRef100_Q9FZ46 Cluster: F6I1.13 protein; n=1; Arabidopsis thaliana\|Rep: F6I1.13 protein - Arabidopsis thaliana (Mouse-ear cress), partial (86%) |
| miR6029 | similar to UniRef100_Q84WK9 Cluster: At4g33985; n=1; Arabidopsis thaliana\|Rep: At4g33985 - Arabidopsis thaliana (Mouse-ear cress), partial (96%) |
| miR6029 | UniRef100_P42027 Cluster: NADH-ubiquinone oxidoreductase 20 kDa subunit, mitochondrial precursor; n=1; Brassica oleracea\|Rep: NADH-ubiquinone oxidoreductase 20 kDa subunit, mitochondrial precursor - Brassica oleracea (Wild cabbage), complete |
| miR6035 | homologue to UniRef100_P48482 Cluster: Serine/threonine-protein phosphatase PP1 isozyme 2; n=1; Arabidopsis thaliana\|Rep: Serine/threonine-protein phosphatase PP1 isozyme 2 - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| miR6035 | homologue to UniRef100_Q93Y22 Cluster: Coatomer subunit delta; n=1; Arabidopsis thaliana\|Rep: Coatomer subunit delta - Arabidopsis thaliana (Mouse-ear cress), partial (97%) |
| miR6035 | similar to UniRef100_Q9FXC5 Cluster: F12A21.27; n=1; Arabidopsis thaliana\|Rep: F12A21.27 - Arabidopsis thaliana (Mouse-ear cress), partial (94%) |
| miR6035 | similar to UniRef100_O81861 Cluster: Chitinase-like protein; n=1; Arabidopsis thaliana\|Rep: Chitinase-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (80%) |
| miR6035 | homologue to UniRef100_Q9LPM3 Cluster: F2J10.10 protein; n=1; Arabidopsis thaliana\|Rep: F2J10.10 protein - Arabidopsis thaliana (Mouse-ear cress), partial (93%) |
| miR6035 | similar to UniRef100_O23154 Cluster: Cytochrome P450-like protein; n=1; Arabidopsis thaliana\|Rep: Cytochrome P450-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (92%) |

# **Supplementary File 8**: Targets of novel DE miRNAs predicted by psRNA target.

| miRNA_Acc. | Target_Desc. |
|---|---|
| bna-N_miR1 | homologue to UniRef100_Q9M4F6 Cluster: SLL3 ORF2 protein; n=1; Brassica napus var. napus\|Rep: SLL3 ORF2 protein - Brassica napus var. napus, complete |
| bna-N_miR1 | UniRef100_Q9XQ96 Cluster: NAD(P)H-quinone oxidoreductase chain 2, chloroplast (EC 1.6.5.-) (NAD(P)H dehydrogenase, chain 2); n=1; Brassica napus\|Rep: NAD(P)H-quinone oxidoreductase chain 2 |
| bna-N_miR1 | similar to UniRef100_Q9SB32 Cluster: SRG1-like protein; n=1; Arabidopsis thaliana\|Rep: SRG1-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (74%) |
| bna-N_miR2 | homologue to UniRef100_Q8L9A9 Cluster: Probable xyloglucan endotransglucosylase/hydrolase protein 8 precursor; n=2; Arabidopsis thaliana\|Rep: Probable xyloglucan endotransglucosylase/hydro |
| bna-N_miR2 | homologue to UniRef100_Q93Y22 Cluster: Coatomer subunit delta; n=1; Arabidopsis thaliana\|Rep: Coatomer subunit delta - Arabidopsis thaliana (Mouse-ear cress), partial (97%) |
| bna-N_miR2 | homologue to UniRef100_Q8GV05 Cluster: Transcription factor TRIPTYCHON; n=1; Arabidopsis thaliana\|Rep: Transcription factor TRIPTYCHON - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR2 | UniRef100_O23733 Cluster: Cysteine synthase (EC 2.5.1.47) (O-acetylserine sulfhydrylase) (O- acetylserine (Thiol)-lyase); n=1; Brassica juncea\|Rep: Cysteine synthase (EC 2.5.1.47) (O-acetylserine su |
| bna-N_miR2 | similar to UniRef100_Q8VZT3 Cluster: Sugar transporter ERD6-like 12; n=1; Arabidopsis thaliana\|Rep: Sugar transporter ERD6-like 12 - Arabidopsis thaliana (Mouse-ear cress), partial (92%) |
| bna-N_miR2 | similar to UniRef100_Q9FJB8 Cluster: Gb\|AAB63610.1; n=1; Arabidopsis thaliana\|Rep: Gb\|AAB63610.1 - Arabidopsis thaliana (Mouse-ear cress), partial (76%) |
| bna-N_miR2 | homologue to UniRef100_Q39315 Cluster: Acyl-CoA-binding protein; n=1; Brassica napus\|Rep: Acyl-CoA-binding protein - Brassica napus (Rape), complete |
| bna-N_miR2 | homologue to UniRef100_Q9FLJ2 Cluster: NAM (No apical meristem)-like protein; n=2; Arabidopsis thaliana\|Rep: NAM (No apical meristem)-like protein - Arabidopsis thaliana (Mouse-ear cress), pa |
| bna-N_miR4 | homologue to UniRef100_Q3E902 Cluster: 40S ribosomal protein S21-2; n=2; Arabidopsis thaliana\|Rep: 40S ribosomal protein S21-2 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR6 | similar to UniRef100_Q84TE6 Cluster: NAC domain-containing protein 21/22; n=2; Arabidopsis thaliana\|Rep: NAC domain-containing protein 21/22 - Arabidopsis thaliana (Mouse-ear cress), partial (9 |
| bna-N_miR6 | homologue to UniRef100_Q9SJV5 Cluster: TATC-like protein; n=1; Arabidopsis thaliana\|Rep: TATC-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (92%) |
| bna-N_miR6 | similar to UniRef100_Q9FPS3 Cluster: Ubiquitin carboxyl-terminal hydrolase 24; n=1; Arabidopsis thaliana\|Rep: Ubiquitin carboxyl-terminal hydrolase 24 - Arabidopsis thaliana (Mouse-ear cress), pa |
| bna-N_miR8 | weakly similar to UniRef100_Q5Z9P7 Cluster: Calcium-dependent protein kinase CPK1 adapter protein 2-like; n=1; Oryza sativa Japonica Group\|Rep: Calcium-dependent protein kinase CPK1 adapter |
| bna-N_miR8 | similar to UniRef100_Q6NLY8 Cluster: HVA22-like protein k; n=1; Arabidopsis thaliana\|Rep: HVA22-like protein k - Arabidopsis thaliana (Mouse-ear cress), partial (82%) |
| bna-N_miR8 | UniRef100_Q6DLS1 Cluster: SCARECROW-like protein; n=1; Brassica napus\|Rep: SCARECROW-like protein - Brassica napus (Rape), complete |
| bna-N_miR9 | homologue to UniRef100_P22953 Cluster: Heat shock cognate 70 kDa protein 1; n=3; Arabidopsis thaliana\|Rep: Heat shock cognate 70 kDa protein 1 - Arabidopsis thaliana (Mouse-ear cress), partial (9 |
| bna-N_miR10 | similar to UniRef100_O82772 Cluster: Beta-glucosidase homolog; n=1; Arabidopsis thaliana\|Rep: Beta-glucosidase homolog - Arabidopsis thaliana (Mouse-ear cress), partial (56%) |
| bna-N_miR11 | homologue to UniRef100_Q9LMK0 Cluster: 60S ribosomal protein L35a-1; n=1; Arabidopsis thaliana\|Rep: 60S ribosomal protein L35a-1 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR12 | similar to UniRef100_Q8LDS6 Cluster: PREDICTED: Brassica napus heat shock 70 kDa protein 6, chloroplastic-like (LOC106440803), transcript variant X2, mRNA |
| bna-N_miR15 | similar to UniRef100_Q96514 Cluster: Cytochrome P450 71B7; n=1; Arabidopsis thaliana\|Rep: Cytochrome P450 71B7 - Arabidopsis thaliana (Mouse-ear cress), partial (61%) |
| bna-N_miR16 | similar to UniRef100_Q9ZSY9 Cluster: Hydroperoxide lyase; n=1; Arabidopsis thaliana\|Rep: Hydroperoxide lyase - Arabidopsis thaliana (Mouse-ear cress), partial (68%) |
| bna-N_miR17 | homologue to UniRef100_Q9FJP9 Cluster: Succinate dehydrogenase [ubiquinone] iron-sulfur subunit 3, mitochondrial precursor; n=1; Arabidopsis thaliana\|Rep: Succinate dehydrogenase [ubiquinor |
| bna-N_miR18 | similar to UniRef100_Q9FJP9 Cluster: Succinate dehydrogenase [ubiquinone] iron-sulfur subunit 3, mitochondrial precursor; n=1; Arabidopsis thaliana\|Rep: Succinate dehydrogenase [ubiquinone] ir |
| bna-N_miR18 | homologue to UniRef100_Q8W4N9 Cluster: Nitrate transporter; n=1; Arabidopsis thaliana\|Rep: Nitrate transporter - Arabidopsis thaliana (Mouse-ear cress), partial (70%) |
| bna-N_miR18 | similar to UniRef100_P50287 Cluster: L-asparaginase 1 precursor (EC 3.5.1.1) (L-asparagine amidohydrolase 1) [Contains: L-asparagine 1 subunit alpha; L-asparaginase 1 subunit beta]; n=2; Arabidop |
| bna-N_miR18 | UniRef100_O23515 Cluster: 60S ribosomal protein L15-1; n=1; Arabidopsis thaliana\|Rep: 60S ribosomal protein L15-1 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR18 | homologue to UniRef100_O49547 Cluster: Phosphoenolpyruvate carboxykinase (ATP) - like protein; n=1; Arabidopsis thaliana\|Rep: Phosphoenolpyruvate carboxykinase (ATP) - like protein - Arabid |
| bna-N_miR18 | similar to UniRef100_Q8VXU6 Cluster: Protein DEHYDRATION-INDUCED 19 homolog 4; n=1; Arabidopsis thaliana\|Rep: Protein DEHYDRATION-INDUCED 19 homolog 4 - Arabidopsis thaliana (Mouse-ea |
| bna-N_miR18 | homologue to UniRef100_Q9LD44 Cluster: Jasmonic acid regulatory protein-like; n=1; Arabidopsis thaliana\|Rep: Jasmonic acid regulatory protein-like - Arabidopsis thaliana (Mouse-ear cress), partia |
| bna-N_miR19 | homologue to UniRef100_Q9LPM3 Cluster: F2J10.10 protein; n=1; Arabidopsis thaliana\|Rep: F2J10.10 protein - Arabidopsis thaliana (Mouse-ear cress), partial (90%) |
| bna-N_miR20 | homologue to homologue to UniRef100_Q9LW76 Cluster: RAS-related GTP-binding protein; n=1; Arabidopsis thaliana\|Rep: RAS-related GTP-binding protein - Arabidopsis thaliana (Mouse-ear cress) |
| bna-N_miR25 | homologue to UniRef100_Q9FEE2 Cluster: Tonneau 2; n=1; Arabidopsis thaliana\|Rep: Tonneau 2 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR25 | homologue to UniRef100_Q8LAP4 Cluster: Contains similarity to MYB-related DNA-binding protein; n=1; Arabidopsis thaliana\|Rep: Contains similarity to MYB-related DNA-binding protein - Arabidop |
| bna-N_miR25 | similar to UniRef100_Q6NLW5 Cluster: At5g48720; n=1; Arabidopsis thaliana\|Rep: At5g48720 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR25 | similar to UniRef100_Q6NLW5 Cluster: At5g48720; n=1; Arabidopsis thaliana\|Rep: At5g48720 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR25 | similar to UniRef100_Q0I5I1 Cluster: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; n=1; Haemophilus somnus 129PT\|Rep: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase - Hae |
| bna-N_miR26 | homologue to UniRef100_UPI0000EB00E4 Cluster: Histone H4; n=1; Canis lupus familiaris\|Rep: Histone H4 - Canis familiaris, partial (98%) |
| bna-N_miR27 | similar to UniRef100_Q94CG2 Cluster: Bet1-like SNARE 1-2; n=2; Arabidopsis thaliana\|Rep: Bet1-like SNARE 1-2 - Arabidopsis thaliana (Mouse-ear cress), partial (51%) |
| bna-N_miR27 | homologue to UniRef100_Q84VW0 Cluster: At4g15930; n=1; Arabidopsis thaliana\|Rep: At4g15930 - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| bna-N_miR28 | homologue to UniRef100_Q84VW0 Cluster: At4g15930; n=1; Arabidopsis thaliana\|Rep: At4g15930 - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| bna-N_miR28 | homologue to UniRef100_Q93W93 Cluster: F-box/Kelch-repeat protein At1g55270; n=1; Arabidopsis thaliana\|Rep: F-box/Kelch-repeat protein At1g55270 - Arabidopsis thaliana (Mouse-ear cress), pa |
| bna-N_miR29 | similar to UniRef100_Q9LK79 Cluster: Emb\|CAB82946.1; n=1; Arabidopsis thaliana\|Rep: Emb\|CAB82946.1 - Arabidopsis thaliana (Mouse-ear cress), partial (55%) |
| bna-N_miR29 | similar to UniRef100_O82798 Cluster: Two-component response regulator ARR4; n=2; Arabidopsis thaliana\|Rep: Two-component response regulator ARR4 - Arabidopsis thaliana (Mouse-ear cress), p |
| bna-N_miR29 | homologue to UniRef100_O48818 Cluster: Expansin-A4 precursor; n=1; Arabidopsis thaliana\|Rep: Expansin-A4 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (55%) |
| bna-N_miR29 | similar to UniRef100_Q94AV9 Cluster: At1g08350/T27G7_4; n=1; Arabidopsis thaliana\|Rep: At1g08350/T27G7_4 - Arabidopsis thaliana (Mouse-ear cress), partial (69%) |
| bna-N_miR29 | homologue to UniRef100_Q9SJT9 Cluster: Coatomer subunit alpha-2; n=1; Arabidopsis thaliana\|Rep: Coatomer subunit alpha-2 - Arabidopsis thaliana (Mouse-ear cress), partial (65%) |
| bna-N_miR29 | homologue to UniRef100_Q9FLJ2 Cluster: NAM (No apical meristem)-like protein; n=2; Arabidopsis thaliana\|Rep: NAM (No apical meristem)-like protein - Arabidopsis thaliana (Mouse-ear cress), pa |
| bna-N_miR30 | homologue to UniRef100_Q42563 Cluster: Adenine phosphoribosyltransferase 2; n=1; Arabidopsis thaliana\|Rep: Adenine phosphoribosyltransferase 2 - Arabidopsis thaliana (Mouse-ear cress), comp |
| bna-N_miR30 | homologue to UniRef100_Q9ZVJ4 Cluster: Peptidyl-prolyl cis-trans isomerase; n=1; Arabidopsis thaliana\|Rep: Peptidyl-prolyl cis-trans isomerase - Arabidopsis thaliana (Mouse-ear cress), partial (98 |
| bna-N_miR32 | homologue to UniRef100_Q8L934 Cluster: Nucleoid DNA-binding-like protein; n=1; Arabidopsis thaliana\|Rep: Nucleoid DNA-binding-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (88 |
| bna-N_miR32 | similar to UniRef100_Q84S20 Cluster: CHP-rich zinc finger protein-like; n=2; Oryza sativa\|Rep: CHP-rich zinc finger protein-like - Oryza sativa subsp. japonica (Rice), partial (52%) |
| bna-N_miR32 | homologue to UniRef100_O49935 Cluster: Sig1 protein; n=1; Sinapis alba\|Rep: Sig1 protein - Sinapis alba (White mustard) (Brassica hirta), partial (98%) |
| bna-N_miR35 | homologue to UniRef100_O23710 Cluster: Proteasome subunit beta type-7-A precursor; n=2; Arabidopsis thaliana\|Rep: Proteasome subunit beta type-7-A precursor - Arabidopsis thaliana (Mouse-e |
| bna-N_miR36 | homologue to UniRef100_A2BPT6 Cluster: 3-oxoacyl-[acyl-carrier protein] reductase; n=1; Prochlorococcus marinus str. AS9601\|Rep: 3-oxoacyl-[acyl-carrier protein] reductase - Prochlorococcus mari |
| bna-N_miR36 | UniRef100_A4URF3 Cluster: Trans-membrane water channel protein; n=1; Brassica juncea\|Rep: Trans-membrane water channel protein - Brassica juncea (Leaf mustard) (Indian mustard), complete |
| bna-N_miR36 | similar to UniRef100_A7NWA5 Cluster: 50S ribosomal protein L33; n=1; Vitis vinifera\|Rep: 50S ribosomal protein L33 - Vitis vinifera (Grape), partial (81%) |
| bna-N_miR37 | similar to UniRef100_Q9SKY8 Cluster: 70kD heat shock protein; n=1; Arabidopsis thaliana\|Rep: 70kD heat shock protein - Arabidopsis thaliana (Mouse-ear cress), partial (58%) |
| bna-N_miR38 | homologue to UniRef100_Q9FT97 Cluster: Alpha-galactosidase-like protein; n=1; Arabidopsis thaliana\|Rep: Alpha-galactosidase-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (91%) |
| bna-N_miR39 | similar to UniRef100_Q9FT97 Cluster: Alpha-galactosidase-like protein; n=1; Arabidopsis thaliana\|Rep: Alpha-galactosidase-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| bna-N_miR39 | similar to UniRef100_Q949Q7 Cluster: Serine carboxypeptidase-like 29 precursor; n=1; Arabidopsis thaliana\|Rep: Serine carboxypeptidase-like 29 precursor - Arabidopsis thaliana (Mouse-ear cress), |
| bna-N_miR40 | similar to UniRef100_Q9ZU70 Cluster: Homeobox-leucine zipper protein ATHB-21; n=1; Arabidopsis thaliana\|Rep: Homeobox-leucine zipper protein ATHB-21 - Arabidopsis thaliana (Mouse-ear cress |
| bna-N_miR40 | UniRef100_Q45W78 Cluster: Ubiquitin fusion protein; n=3; core eudicotyledons\|Rep: Ubiquitin fusion protein - Arachis hypogaea (Peanut), complete |
| bna-N_miR41 | similar to UniRef100_Q42431 Cluster: Oleosin 20.3 kDa; n=1; Arabidopsis thaliana\|Rep: Oleosin 20.3 kDa - Arabidopsis thaliana (Mouse-ear cress), partial (60%) |
| bna-N_miR41 | homologue to UniRef100_Q9LVC5 Cluster: Apospory-associated protein C; n=1; Arabidopsis thaliana\|Rep: Apospory-associated protein C - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR41 | homologue to UniRef100_A1YMZ0 Cluster: CHL-CPN10; n=1; Brassica rapa\|Rep: CHL-CPN10 - Brassica campestris (Field mustard), complete |
| bna-N_miR41 | homologue to UniRef100_O48814 Cluster: Serine/threonine-protein kinase BIK1; n=1; Arabidopsis thaliana\|Rep: Serine/threonine-protein kinase BIK1 - Arabidopsis thaliana (Mouse-ear cress), parti |
| bna-N_miR41 | homologue to UniRef100_Q9FT97 Cluster: Alpha-galactosidase-like protein; n=1; Arabidopsis thaliana\|Rep: Alpha-galactosidase-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (91%) |
| bna-N_miR41 | similar to UniRef100_Q9SRH4 Cluster: Probable pectate lyase 7 precursor; n=2; Arabidopsis thaliana\|Rep: Probable pectate lyase 7 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (96%) |
| bna-N_miR42 | homologue to UniRef100_P42697 Cluster: Dynamin-related protein 1A; n=3; Arabidopsis thaliana\|Rep: Dynamin-related protein 1A - Arabidopsis thaliana (Mouse-ear cress), partial (74%) |
| bna-N_miR42 | similar to UniRef100_Q56Z38 Cluster: Pectate lyase like protein; n=1; Arabidopsis thaliana\|Rep: Pectate lyase like protein - Arabidopsis thaliana (Mouse-ear cress), partial (70%) |
| bna-N_miR42 | UniRef100_Q08112 Cluster: 40S ribosomal protein S15-1; n=1; Arabidopsis thaliana\|Rep: 40S ribosomal protein S15-1 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR43 | homologue to UniRef100_Q9C6Y3 Cluster: Cyclin-A1-1; n=1; Arabidopsis thaliana\|Rep: Cyclin-A1-1 - Arabidopsis thaliana (Mouse-ear cress), partial (60%) |
| bna-N_miR43 | homologue to UniRef100_Q9FI61 Cluster: Ubiquitin carrier protein; n=1; Arabidopsis thaliana\|Rep: Ubiquitin carrier protein - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR43 | homologue to UniRef100_Q9FLZ4 Cluster: Cyclin-dependent protein kinase-like protein; n=1; Arabidopsis thaliana\|Rep: Cyclin-dependent protein kinase-like protein - Arabidopsis thaliana (Mouse- |
| bna-N_miR44 | homologue to UniRef100_O64431 Cluster: Lipid transfer protein; n=1; Brassica rapa\|Rep: Lipid transfer protein - Brassica campestris (Field mustard), complete |
| bna-N_miR44 | similar to UniRef100_O82197 Cluster: Copia-like retroelement pol polyprotein; n=1; Arabidopsis thaliana\|Rep: Copia-like retroelement pol polyprotein - Arabidopsis thaliana (Mouse-ear cress), part |
| bna-N_miR44 | homologue to UniRef100_Q39402 Cluster: Br FatA1; n=1; Brassica rapa\|Rep: Br FatA1 - Brassica campestris (Field mustard), complete |
| bna-N_miR45 | similar to UniRef100_Q9SJT9 Cluster: Coatomer subunit alpha-2; n=1; Arabidopsis thaliana\|Rep: Coatomer subunit alpha-2 - Arabidopsis thaliana (Mouse-ear cress), partial (52%) |
| bna-N_miR46 | homologue to UniRef100_Q42438 Cluster: Calcium-dependent protein kinase; n=3; Arabidopsis thaliana\|Rep: Calcium-dependent protein kinase - Arabidopsis thaliana (Mouse-ear cress), partial (98 |
| bna-N_miR46 | similar to UniRef100_Q9FNN2 Cluster: WD-repeat protein-like; n=1; Arabidopsis thaliana\|Rep: WD-repeat protein-like - Arabidopsis thaliana (Mouse-ear cress), partial (53%) |
| bna-N_miR46 | similar to UniRef100_O82244 Cluster: Not56-like protein; n=2; Arabidopsis thaliana\|Rep: Not56-like protein - Arabidopsis thaliana (Mouse-ear cress), partial (89%) |
| bna-N_miR46 | homologue to UniRef100_Q9S9T7 Cluster: Vacuolar protein sorting-associated protein 28 homolog 1; n=1; Arabidopsis thaliana\|Rep: Vacuolar protein sorting-associated protein 28 homolog 1 - Arabi |
| bna-N_miR46 | weakly similar to UniRef100_Q9WVJ4 Cluster: Synaptojanin-2-binding protein; n=2; Rattus norvegicus\|Rep: Synaptojanin-2-binding protein - Rattus norvegicus (Rat), partial (14%) |

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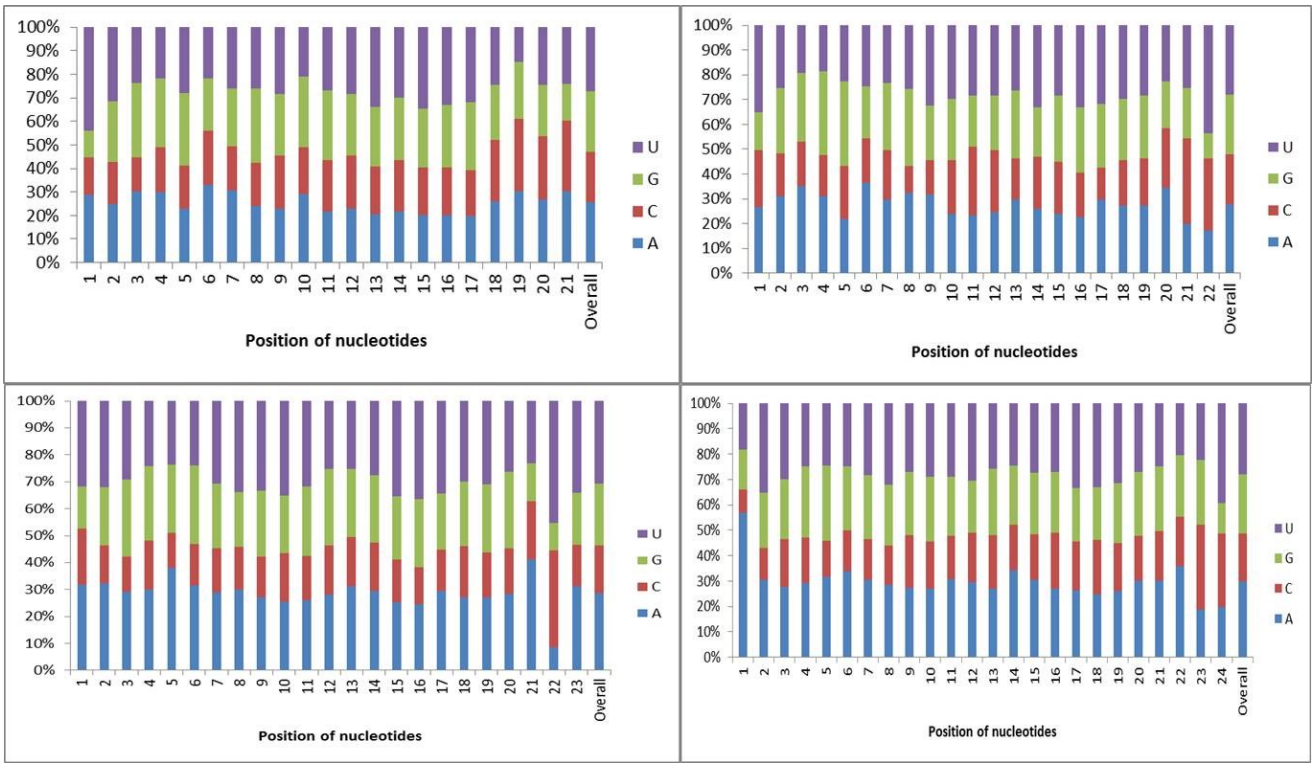
|---|---|
| bna-N_miR46 | weakly similar to UniRef100_UPI000065DF04 Cluster: Beta-hexosaminidase beta chain precursor (EC 3.2.1.52) (N-acetyl-beta- glucosaminidase) (Beta-N-acetylhexosaminidase) (Hexosaminidase B) ( |
| bna-N_miR46 | homologue to UniRef100_Q93W01 Cluster: At2g01080/F23H14.5; n=1; Arabidopsis thaliana\|Rep: At2g01080/F23H14.5 - Arabidopsis thaliana (Mouse-ear cress), partial (89%) |
| bna-N_miR46 | homologue to UniRef100_P18064 Cluster: Guanine nucleotide-binding protein alpha-1 subunit; n=1; Arabidopsis thaliana\|Rep: Guanine nucleotide-binding protein alpha-1 subunit - Arabidopsis tha |
| bna-N_miR47 | homologue to UniRef100_Q9ST43 Cluster: Pleckstrin homology domain-containing protein 1; n=1; Arabidopsis thaliana\|Rep: Pleckstrin homology domain-containing protein 1 - Arabidopsis thaliana |
| bna-N_miR48 | UniRef100_A1YSR1 Cluster: Dihydroflavonol 4-reductase; n=1; Brassica juncea\|Rep: Dihydroflavonol 4-reductase - Brassica juncea (Leaf mustard) (Indian mustard), complete |
| bna-N_miR50 | homologue to UniRef100_Q9FN15 Cluster: Gb\|AAC18972.1; n=1; Arabidopsis thaliana\|Rep: Gb\|AAC18972.1 - Arabidopsis thaliana (Mouse-ear cress), partial (80%) |
| bna-N_miR51 | similar to UniRef100_Q1PF10 Cluster: Glycoside hydrolase family 28 protein/polygalacturonase family protein; n=3; Arabidopsis thaliana\|Rep: Glycoside hydrolase family 28 protein/polygalacturona |
| bna-N_miR51 | homologue to UniRef100_Q9SRX3 Cluster: Endoglucanase 1 precursor; n=1; Arabidopsis thaliana\|Rep: Endoglucanase 1 precursor - Arabidopsis thaliana (Mouse-ear cress), partial (95%) |
| bna-N_miR51 | homologue to UniRef100_P28186 Cluster: Ras-related protein ARA-3; n=1; Arabidopsis thaliana\|Rep: Ras-related protein ARA-3 - Arabidopsis thaliana (Mouse-ear cress), partial (88%) |
| bna-N_miR51 | similar to UniRef100_Q84R26 Cluster: 50S ribosomal protein L34; n=1; Arabidopsis thaliana\|Rep: 50S ribosomal protein L34 - Arabidopsis thaliana (Mouse-ear cress), partial (63%) |
| bna-N_miR51 | similar to UniRef100_Q9SEU8 Cluster: Thioredoxin M-type 2, chloroplast precursor; n=2; Arabidopsis thaliana\|Rep: Thioredoxin M-type 2, chloroplast precursor - Arabidopsis thaliana (Mouse-ear cre |
| bna-N_miR51 | similar to UniRef100_UPI00015057C4 Cluster: amino acid permease; n=1; Arabidopsis thaliana\|Rep: amino acid permease - Arabidopsis thaliana, partial (96%) |
| bna-N_miR52 | UniRef100_Q93Y50 Cluster: Biotin carboxylase; n=1; Brassica napus\|Rep: Biotin carboxylase - Brassica napus (Rape), complete |
| bna-N_miR53 | similar to UniRef100_Q1PFA4 Cluster: MADS-box family protein; n=2; Arabidopsis thaliana\|Rep: MADS-box family protein - Arabidopsis thaliana (Mouse-ear cress), partial (93%) |
| bna-N_miR57 | UniRef100_Q6DLS1 Cluster: SCARECROW-like protein; n=1; Brassica napus\|Rep: SCARECROW-like protein - Brassica napus (Rape), complete |
| bna-N_miR57 | homologue to UniRef100_Q9SFC6 Cluster: Rho GDP-dissociation inhibitor 1; n=2; Arabidopsis thaliana\|Rep: Rho GDP-dissociation inhibitor 1 - Arabidopsis thaliana (Mouse-ear cress), partial (69%) |
| bna-N_miR57 | weakly similar to UniRef100_Q4YYH0 Cluster: BIR; n=1; Plasmodium berghei\|Rep: BIR - Plasmodium berghei, partial (76%) |
| bna-N_miR57 | homologue to UniRef100_Q8LEM7 Cluster: Calcineurin B-like protein 3; n=1; Arabidopsis thaliana\|Rep: Calcineurin B-like protein 3 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR58 | homologue to UniRef100_Q8LKQ7 Cluster: VTC2; n=1; Arabidopsis thaliana\|Rep: VTC2 - Arabidopsis thaliana (Mouse-ear cress), partial (94%) |
| bna-N_miR58 | homologue to UniRef100_Q8LBH9 Cluster: Cytochrome c biogenesis protein; n=1; Arabidopsis thaliana\|Rep: Cytochrome c biogenesis protein - Arabidopsis thaliana (Mouse-ear cress), partial (97%) |
| bna-N_miR59 | homologue to UniRef100_Q9FPH3 Cluster: AT3g04520; n=1; Arabidopsis thaliana\|Rep: AT3g04520 - Arabidopsis thaliana (Mouse-ear cress), partial (50%) |
| bna-N_miR60 | weakly similar to UniRef100_Q4TTZ9 Cluster: Rapid alkalinization factor 1; n=1; Brassica oleracea var. botrytis\|Rep: Rapid alkalinization factor 1 - Brassica oleracea var. botrytis (Cauliflower), partial (8 |
| bna-N_miR63 | similar to UniRef100_Q49KZ4 Cluster: NADH-plastoquinone oxidoreductase subunit K; n=1; Eucalyptus globulus subsp. globulus\|Rep: NADH-plastoquinone oxidoreductase subunit K - Eucalyptus glo |
| bna-N_miR65 | homologue to UniRef100_Q9FXD8 Cluster: Probable pectate lyase 5 precursor; n=1; Arabidopsis thaliana\|Rep: Probable pectate lyase 5 precursor - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR65 | similar to UniRef100_Q8L557 Cluster: EMB514; n=2; Arabidopsis thaliana\|Rep: EMB514 - Arabidopsis thaliana (Mouse-ear cress), partial (82%) |
| bna-N_miR65 | similar to UniRef100_Q39132 Cluster: Major latex protein type1; n=1; Arabidopsis thaliana\|Rep: Major latex protein type1 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR65 | similar to UniRef100_Q8L9J9 Cluster: Probable carbohydrate esterase At4g34215; n=1; Arabidopsis thaliana\|Rep: Probable carbohydrate esterase At4g34215 - Arabidopsis thaliana (Mouse-ear cress), |
| bna-N_miR65 | UniRef100_Q84JA9 Cluster: NAD-dependent isocitrate dehydrogenase alpha subunit; n=1; Brassica napus\|Rep: NAD-dependent isocitrate dehydrogenase alpha subunit - Brassica napus (Rape), parti |
| bna-N_miR66 | homologue to UniRef100_Q84JA9 Cluster: NAD-dependent isocitrate dehydrogenase alpha subunit; n=1; Brassica napus\|Rep: NAD-dependent isocitrate dehydrogenase alpha subunit - Brassica napu |
| bna-N_miR66 | homologue to UniRef100_Q945K7 Cluster: Isocitrate dehydrogenase [NAD] catalytic subunit 5, mitochondrial precursor (EC 1.1.1.41) (Isocitric dehydrogenase 5) (NAD(+)-specific ICDH 5); n=1; Arabid |
| bna-N_miR68 | homologue to UniRef100_Q945K7 Cluster: Isocitrate dehydrogenase [NAD] catalytic subunit 5, mitochondrial precursor (EC 1.1.1.41) (Isocitric dehydrogenase 5) (NAD(+)-specific ICDH 5); n=1; Arabid |
| bna-N_miR68 | homologue to UniRef100_P13851 Cluster: Chlorophyll a-b binding protein 1, chloroplast precursor; n=1; Sinapis alba\|Rep: Chlorophyll a-b binding protein 1, chloroplast precursor - Sinapis alba (Whit |
| bna-N_miR68 | weakly similar to UniRef100_Q4TTZ9 Cluster: Rapid alkalinization factor 1; n=1; Brassica oleracea var. botrytis\|Rep: Rapid alkalinization factor 1 - Brassica oleracea var. botrytis (Cauliflower), partial (8 |
| bna-N_miR69 | homologue to UniRef100_P41507 Cluster: Anther-specific protein BCP1; n=1; Brassica rapa\|Rep: Anther-specific protein BCP1 - Brassica campestris (Field mustard), partial (97%) |
| bna-N_miR69 | homologue to UniRef100_Q9FXD8 Cluster: Probable pectate lyase 5 precursor; n=1; Arabidopsis thaliana\|Rep: Probable pectate lyase 5 precursor - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR69 | homologue to UniRef100_A0MKC8 Cluster: Ubiquitin extension protein; n=2; core eudicotyledons\|Rep: Ubiquitin extension protein - Capsicum annuum (Bell pepper), partial (87%) |
| bna-N_miR69 | homologue to UniRef100_Q2A9Q7 Cluster: Hydrolase, NUDIX family protein; n=1; Brassica oleracea\|Rep: Hydrolase, NUDIX family protein - Brassica oleracea (Wild cabbage), partial (95%) |
| bna-N_miR69 | weakly similar to UniRef100_Q4K366 Cluster: Flippase Wzx; n=3; Streptococcus pneumoniae\|Rep: Flippase Wzx - Streptococcus pneumoniae, partial (4%) |
| bna-N_miR69 | homologue to UniRef100_UPI000034F513 Cluster: hydrolase, alpha/beta fold family protein; n=1; Arabidopsis thaliana\|Rep: hydrolase, alpha/beta fold family protein - Arabidopsis thaliana, partial ( |
| bna-N_miR69 | homologue to UniRef100_P48482 Cluster: Serine/threonine-protein phosphatase PP1 isozyme 2; n=1; Arabidopsis thaliana\|Rep: Serine/threonine-protein phosphatase PP1 isozyme 2 - Arabidopsis t |
| bna-N_miR69 | similar to UniRef100_Q8L557 Cluster: EMB514; n=2; Arabidopsis thaliana\|Rep: EMB514 - Arabidopsis thaliana (Mouse-ear cress), partial (82%) |
| bna-N_miR69 | homologue to UniRef100_Q704T1 Cluster: SGT1-like protein; n=1; Brassica oleracea\|Rep: SGT1-like protein - Brassica oleracea (Wild cabbage), complete |
| bna-N_miR69 | homologue to UniRef100_Q9FFT4 Cluster: Pyruvate decarboxylase; n=3; Arabidopsis thaliana\|Rep: Pyruvate decarboxylase - Arabidopsis thaliana (Mouse-ear cress), partial (55%) |
| bna-N_miR69 | similar to UniRef100_Q8GY25 Cluster: WUSCHEL-related homeobox 12; n=1; Arabidopsis thaliana\|Rep: WUSCHEL-related homeobox 12 - Arabidopsis thaliana (Mouse-ear cress), partial (90%) |
| bna-N_miR70 | homologue to UniRef100_Q7XYW0 Cluster: Seed specific protein Bn15D33A; n=1; Brassica napus\|Rep: Seed specific protein Bn15D33A - Brassica napus (Rape), complete |
| bna-N_miR71 | similar to UniRef100_Q941B1 Cluster: At1g69520/F10D13_17; n=1; Arabidopsis thaliana\|Rep: At1g69520/F10D13_17 - Arabidopsis thaliana (Mouse-ear cress), partial (60%) |
| bna-N_miR72 | homologue to UniRef100_Q9FUB2 Cluster: PRLI-interacting factor K; n=2; Arabidopsis thaliana\|Rep: PRLI-interacting factor K - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| bna-N_miR73 | homologue to UniRef100_Q9SEU4 Cluster: Serine/arginine-rich protein; n=1; Arabidopsis thaliana\|Rep: Serine/arginine-rich protein - Arabidopsis thaliana (Mouse-ear cress), partial (38%) |
| bna-N_miR73 | homologue to UniRef100_Q9SZJ5 Cluster: Serine hydroxymethyltransferase, mitochondrial precursor; n=3; Arabidopsis thaliana\|Rep: Serine hydroxymethyltransferase, mitochondrial precursor - Ar |
| bna-N_miR75 | similar to UniRef100_Q9M9V0 Cluster: F6A14.10 protein; n=1; Arabidopsis thaliana\|Rep: F6A14.10 protein - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR75 | similar to UniRef100_Q04980 Cluster: Low-temperature-induced 65 kDa protein; n=1; Arabidopsis thaliana\|Rep: Low-temperature-induced 65 kDa protein - Arabidopsis thaliana (Mouse-ear cress), p |
| bna-N_miR77 | UniRef100_P43402 Cluster: Metallothionein-like protein LSC54; n=2; Brassica\|Rep: Metallothionein-like protein LSC54 - Brassica napus (Rape), complete |
| bna-N_miR78 | homologue to UniRef100_P48482 Cluster: Serine/threonine-protein phosphatase PP1 isozyme 2; n=1; Arabidopsis thaliana\|Rep: Serine/threonine-protein phosphatase PP1 isozyme 2 - Arabidopsis t |
| bna-N_miR78 | similar to UniRef100_Q9LVW0 Cluster: Similarity to stomatin like protein; n=1; Arabidopsis thaliana\|Rep: Similarity to stomatin like protein - Arabidopsis thaliana (Mouse-ear cress), partial (75%) |
| bna-N_miR79 | homologue to UniRef100_Q8LEM7 Cluster: Calcineurin B-like protein 3; n=1; Arabidopsis thaliana\|Rep: Calcineurin B-like protein 3 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR79 | homologue to UniRef100_Q8LKQ7 Cluster: VTC2; n=1; Arabidopsis thaliana\|Rep: VTC2 - Arabidopsis thaliana (Mouse-ear cress), partial (94%) |
| bna-N_miR79 | homologue to UniRef100_Q9LIB5 Cluster: GATA transcription factor 17; n=2; Arabidopsis thaliana\|Rep: GATA transcription factor 17 - Arabidopsis thaliana (Mouse-ear cress), partial (70%) |
| bna-N_miR79 | weakly similar to UniRef100_A7QVK0 Cluster: Chromosome chr16 scaffold_189, whole genome shotgun sequence; n=1; Vitis vinifera\|Rep: Chromosome chr16 scaffold_189, whole genome shotgun s |
| bna-N_miR80 | homologue to UniRef100_Q7Y1Y1 Cluster: Nonsymbiotic hemoglobin; n=1; Raphanus sativus\|Rep: Nonsymbiotic hemoglobin - Raphanus sativus (Radish), partial (81%) |
| bna-N_miR81 | UniRef100_Q7Y1Y1 Cluster: Nonsymbiotic hemoglobin; n=1; Raphanus sativus\|Rep: Nonsymbiotic hemoglobin - Raphanus sativus (Radish), complete |
| bna-N_miR81 | similar to UniRef100_UPI000150578C Cluster: AtRABA6b (Arabidopsis Rab GTPase homolog A6b); GTP binding; n=1; Arabidopsis thaliana\|Rep: AtRABA6b (Arabidopsis Rab GTPase homolog A6b); GTP |
| bna-N_miR81 | homologue to UniRef100_Q93ZB2 Cluster: Ent-kaurene oxidase; n=1; Arabidopsis thaliana\|Rep: Ent-kaurene oxidase - Arabidopsis thaliana (Mouse-ear cress), partial (98%) |
| bna-N_miR81 | homologue to UniRef100_Q9FJT7 Cluster: Pollen specific protein SF21; n=1; Arabidopsis thaliana\|Rep: Pollen specific protein SF21 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR81 | weakly similar to UniRef100_A8VDY7 Cluster: MJ0042 family finger-like protein; n=1; Anaeromyxobacter sp. K\|Rep: MJ0042 family finger-like protein - Anaeromyxobacter sp. K, partial (4%) |
| bna-N_miR81 | similar to UniRef100_Q8W034 Cluster: Ribonucleoprotein 1; n=1; Arabidopsis thaliana\|Rep: Ribonucleoprotein 1 - Arabidopsis thaliana (Mouse-ear cress), partial (61%) |
| bna-N_miR81 | homologue to UniRef100_P39867 Cluster: Nitrate reductase [NADH], clone PBNBR1405; n=1; Brassica napus\|Rep: Nitrate reductase [NADH], clone PBNBR1405 - Brassica napus (Rape), complete |
| bna-N_miR82 | similar to UniRef100_Q9ASZ6 Cluster: AT4g35320/F23E12_120; n=2; Arabidopsis thaliana\|Rep: AT4g35320/F23E12_120 - Arabidopsis thaliana (Mouse-ear cress), partial (50%) |
| bna-N_miR82 | homologue to UniRef100_Q9M9W3 Cluster: Serine/threonine-protein phosphatase PP1 isozyme 9; n=1; Arabidopsis thaliana\|Rep: Serine/threonine-protein phosphatase PP1 isozyme 9 - Arabidopsi |
| bna-N_miR84 | similar to UniRef100_Q9CAN4 Cluster: F-box protein PP2-A11; n=1; Arabidopsis thaliana\|Rep: F-box protein PP2-A11 - Arabidopsis thaliana (Mouse-ear cress), partial (96%) |
| bna-N_miR84 | homologue to UniRef100_Q9M339 Cluster: 40S ribosomal protein S3-2; n=1; Arabidopsis thaliana\|Rep: 40S ribosomal protein S3-2 - Arabidopsis thaliana (Mouse-ear cress), complete |
| bna-N_miR84 | similar to UniRef100_Q39100 Cluster: ExtA protein; n=1; Arabidopsis thaliana\|Rep: ExtA protein - Arabidopsis thaliana (Mouse-ear cress), partial (86%) |
| bna-N_miR85 | homologue to UniRef100_Q9FEA2 Cluster: Glutamyl-tRNA synthetase; n=2; Arabidopsis thaliana\|Rep: Glutamyl-tRNA synthetase - Arabidopsis thaliana (Mouse-ear cress), partial (95%) |

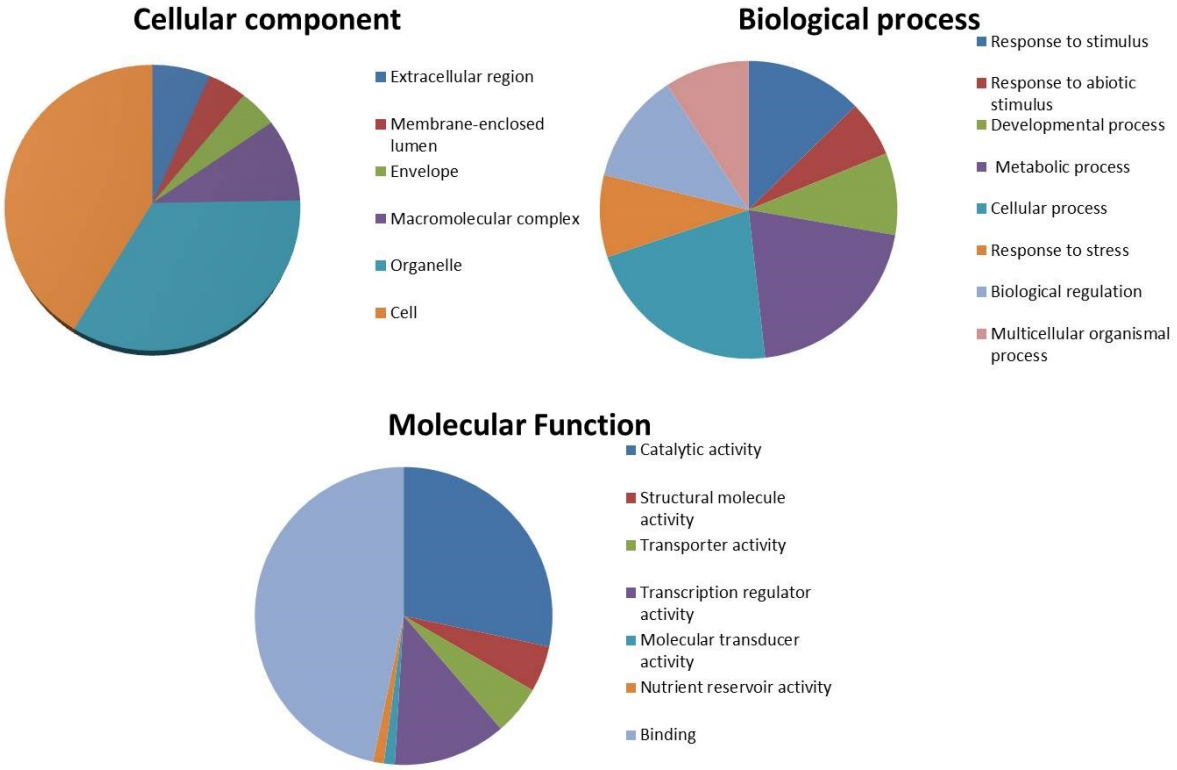
**Supplementary File 9**: Enrichment analysis of GO terms of miRNA targets.

| Category | Term | Count | % | PValue | Genes | List Total | Pop Hits | Pop Total | Fold Enric | Bonferron | Benjamini | FDR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GOTERM_BP_FAT | GO:0006091~generation of precursor metabolites an | 22 | 5.034325 | 0.001278 | UNIREF100_Q9C5I1, UNIREF100_Q9SZ | 263 | 540 | 13998 | 2.168399 | 0.616155 | 0.616155 | 1.926552 |
| GOTERM_BP_FAT | GO:0044271~nitrogen compound biosynthetic proce | 19 | 4.347826 | 0.007086 | UNIREF100_Q8L493, UNIREF100_Q9FV | 263 | 506 | 13998 | 1.998542 | 0.995139 | 0.446684 | 10.25657 |
| GOTERM_BP_FAT | GO:0009266~response to temperature stimulus | 16 | 3.661327 | 0.009188 | UNIREF100_Q9LJI5, UNIREF100_Q9ZU2 | 263 | 404 | 13998 | 2.107894 | 0.999006 | 0.437918 | 13.1037 |
| GOTERM_BP_FAT | GO:0009409~response to cold | 14 | 3.203661 | 0.010282 | UNIREF100_Q9SZJ5, UNIREF100_P229 | 263 | 354 | 13998 | 2.104917 | 0.999995 | 0.584488 | 22.10447 |
| GOTERM_BP_FAT | GO:0006979~response to oxidative stress | 13 | 2.974828 | 0.00149 | UNIREF100_P22953, UNIREF100_Q9SZ | 263 | 233 | 13998 | 2.969598 | 0.67269 | 0.31084 | 2.243507 |
| GOTERM_BP_FAT | GO:0034621~cellular macromolecular complex subur | 13 | 2.974828 | 0.007946 | UNIREF100_Q9LYK8, UNIREF100_Q9M | 263 | 287 | 13998 | 2.410858 | 0.99746 | 0.419129 | 11.43219 |
| GOTERM_BP_FAT | GO:0015672~monovalent inorganic cation transport | 11 | 2.517162 | 0.01021 | UNIREF100_Q8VWK4, UNIREF100_Q9( | 263 | 226 | 13998 | 2.590565 | 0.999541 | 0.446395 | 14.45889 |
| GOTERM_BP_FAT | GO:0006119~oxidative phosphorylation | 10 | 2.28833 | 0.003053 | UNIREF100_Q96253, UNIREF100_Q814 | 263 | 159 | 13998 | 3.347442 | 0.898776 | 0.367506 | 4.546756 |
| GOTERM_BP_FAT | GO:0015986~ATP synthesis coupled proton transport | 7 | 1.601831 | 0.006818 | UNIREF100_Q96253, UNIREF100_Q822 | 263 | 90 | 13998 | 4.13967 | 0.99405 | 0.472997 | 9.88741 |
| GOTERM_BP_FAT | GO:0015985~energy coupled proton transport, down | 6 | 1.372998 | 0.004859 | UNIREF100_Q96253, UNIREF100_Q822 | 263 | 59 | 13998 | 5.412644 | 0.973973 | 0.455617 | 7.144666 |
| GOTERM_BP_FAT | GO:0009100~glycoprotein metabolic process | 6 | 1.372998 | 0.004859 | UNIREF100_Q96253, UNIREF100_Q822 | 263 | 59 | 13998 | 5.412644 | 0.973973 | 0.455617 | 7.144666 |
| GOTERM_BP_FAT | GO:0034220~ion transmembrane transport | 6 | 1.372998 | 0.006424 | UNIREF100_Q8GXG1, UNIREF100_Q0M | 263 | 63 | 13998 | 5.068984 | 0.991989 | 0.498206 | 9.341234 |
| | | | | | | | | | | | | |
| Category | Term | Count | % | PValue | Genes | List Total | Pop Hits | Pop Total | Fold Enric | Bonferron | Benjamini | FDR |
| GOTERM_CC_FAT | GO:0005886~plasma membrane | 61 | 13.95881 | 6.51E-04 | UNIREF100_Q8W553, UNIREF100_P42 | 249 | 2228 | 13779 | 1.515071 | 0.117477 | 0.008887 | 0.807092 |
| GOTERM_CC_FAT | GO:0043232~intracellular non-membrane-bounded ( | 50 | 11.44165 | 8.09E-09 | UNIREF100_Q9FNP8, UNIREF100_P427 | 249 | 1144 | 13779 | 2.41859 | 1.55E-06 | 3.88E-07 | 1.01E-05 |
| GOTERM_CC_FAT | GO:0043228~non-membrane-bounded organelle | 50 | 11.44165 | 8.09E-09 | UNIREF100_Q9FNP8, UNIREF100_P427 | 249 | 1144 | 13779 | 2.41859 | 1.55E-06 | 3.88E-07 | 1.01E-05 |
| GOTERM_CC_FAT | GO:0005829~cytosol | 35 | 8.009153 | 1.59E-07 | UNIREF100_Q9FNP8, UNIREF100_P427 | 249 | 708 | 13779 | 2.735603 | 3.05E-05 | 4.36E-06 | 1.98E-04 |
| GOTERM_CC_FAT | GO:0030529~ribonucleoprotein complex | 34 | 7.78032 | 1.42E-07 | UNIREF100_Q9FNP8, UNIREF100_P427 | 249 | 671 | 13779 | 2.803979 | 2.72E-05 | 4.54E-06 | 1.77E-04 |
| GOTERM_CC_FAT | GO:0031090~organelle membrane | 33 | 7.551487 | 5.74E-05 | UNIREF100_Q39195, UNIREF100_Q9FC | 249 | 849 | 13779 | 2.150922 | 0.010952 | 0.001001 | 0.071383 |
| GOTERM_CC_FAT | GO:0005840~ribosome | 31 | 7.093822 | 1.64E-09 | UNIREF100_Q9FNP8, UNIREF100_Q9FI | 249 | 470 | 13779 | 3.64991 | 3.16E-07 | 1.58E-07 | 2.05E-06 |
| GOTERM_CC_FAT | GO:0022626~cytosolic ribosome | 28 | 6.407323 | 1.91E-11 | UNIREF100_Q9FNP8, UNIREF100_Q9FI | 249 | 317 | 13779 | 4.887842 | 3.67E-09 | 3.67E-09 | 2.38E-08 |
| GOTERM_CC_FAT | GO:0005773~vacuole | 28 | 6.407323 | 3.75E-05 | UNIREF100_P42814, UNIREF100_Q9FN | 249 | 643 | 13779 | 2.409714 | 0.007172 | 7.19E-04 | 0.04666 |
| GOTERM_CC_FAT | GO:0031967~organelle envelope | 27 | 6.17849 | 9.43E-04 | UNIREF100_P27140, UNIREF100_Q9ZU | 249 | 745 | 13779 | 2.005515 | 0.165697 | 0.012005 | 1.167839 |
| GOTERM_CC_FAT | GO:0031975~envelope | 27 | 6.17849 | 0.001046 | UNIREF100_P27140, UNIREF100_Q9ZU | 249 | 751 | 13779 | 1.989492 | 0.182053 | 0.011751 | 1.294649 |
| GOTERM_CC_FAT | GO:0005618~cell wall | 25 | 5.720824 | 2.81E-04 | UNIREF100_P22953, UNIREF100_Q8L9/ | 249 | 611 | 13779 | 2.264212 | 0.052503 | 0.004484 | 0.349105 |
| GOTERM_CC_FAT | GO:0030312~external encapsulating structure | 25 | 5.720824 | 3.48E-04 | UNIREF100_P22953, UNIREF100_Q8L9/ | 249 | 620 | 13779 | 2.231345 | 0.064641 | 0.005127 | 0.432388 |
| GOTERM_CC_FAT | GO:0044445~cytosolic part | 23 | 5.263158 | 3.81E-09 | UNIREF100_Q9FNP8, UNIREF100_Q9FI | 249 | 271 | 13779 | 4.696528 | 7.32E-07 | 2.44E-07 | 4.75E-06 |
| GOTERM_CC_FAT | GO:0043233~organelle lumen | 23 | 5.263158 | 0.003958 | UNIREF100_Q39195, UNIREF100_Q9SZ | 249 | 659 | 13779 | 1.931349 | 0.533033 | 0.037359 | 4.81795 |
| GOTERM_CC_FAT | GO:0070013~intracellular organelle lumen | 23 | 5.263158 | 0.003958 | UNIREF100_Q39195, UNIREF100_Q9SZ | 249 | 659 | 13779 | 1.931349 | 0.533033 | 0.037359 | 4.81795 |
| GOTERM_CC_FAT | GO:0031974~membrane-enclosed lumen | 23 | 5.263158 | 0.004552 | UNIREF100_Q39195, UNIREF100_Q9SZ | 249 | 667 | 13779 | 1.908184 | 0.583538 | 0.040854 | 5.521815 |
| GOTERM_CC_FAT | GO:0033279~ribosomal subunit | 22 | 5.034325 | 8.07E-08 | UNIREF100_Q9FNP8, UNIREF100_Q9FI | 249 | 294 | 13779 | 4.14089 | 1.55E-05 | 3.10E-06 | 1.00E-04 |
| GOTERM_CC_FAT | GO:0005783~endoplasmic reticulum | 17 | 3.89016 | 0.006818 | UNIREF100_Q94BY2, UNIREF100_Q9LN | 249 | 446 | 13779 | 2.109271 | 0.731155 | 0.053263 | 8.165405 |
| GOTERM_CC_FAT | GO:0005730~nucleolus | 16 | 3.661327 | 0.001015 | UNIREF100_P22953, UNIREF100_Q6AV | 249 | 332 | 13779 | 2.66686 | 0.177096 | 0.012108 | 1.25597 |
| GOTERM_CC_FAT | GO:0022625~cytosolic large ribosomal subunit | 15 | 3.432494 | 1.88E-07 | UNIREF100_P42791, UNIREF100_Q9FE | 249 | 138 | 13779 | 6.014929 | 3.61E-05 | 4.52E-06 | 2.34E-04 |
| GOTERM_CC_FAT | GO:0015934~large ribosomal subunit | 15 | 3.432494 | 2.94E-06 | UNIREF100_P42791, UNIREF100_Q9FE | 249 | 173 | 13779 | 4.798036 | 5.65E-04 | 6.28E-05 | 0.003665 |
| GOTERM_CC_FAT | GO:0044429~mitochondrial part | 15 | 3.432494 | 0.00155 | UNIREF100_Q9SZJ5, UNIREF100_Q9ZU | 249 | 311 | 13779 | 2.669004 | 0.257507 | 0.016405 | 1.912171 |
| GOTERM_CC_FAT | GO:0031966~mitochondrial membrane | 11 | 2.517162 | 0.008111 | UNIREF100_Q96253, UNIREF100_Q56X | 249 | 227 | 13779 | 2.681546 | 0.790636 | 0.060631 | 9.642484 |
| GOTERM_CC_FAT | GO:0016469~proton-transporting two-sector ATPase | 6 | 1.372998 | 0.005833 | UNIREF100_Q96253, UNIREF100_Q822 | 249 | 64 | 13779 | 5.187877 | 0.674792 | 0.047665 | 7.024978 |
| GOTERM_CC_FAT | GO:0045259~proton-transporting ATP synthase comp | 5 | 1.144165 | 0.005139 | UNIREF100_Q96253, UNIREF100_Q822 | 249 | 39 | 13779 | 7.094532 | 0.628118 | 0.043967 | 6.212897 |
| GOTERM_CC_FAT | GO:0005753~mitochondrial proton-transporting ATP | 4 | 0.915332 | 0.002712 | UNIREF100_Q96253, UNIREF100_P834 | 249 | 16 | 13779 | 13.83434 | 0.406319 | 0.02707 | 3.324559 |
| | | | | | | | | | | | | |
| GOTERM_MF_FAT | GO:0005198~structural molecule activity | 32 | 7.322654 | 2.84E-08 | UNIREF100_Q9FNP8, UNIREF100_Q9FI | 279 | 538 | 14806 | 3.156467 | 1.14E-05 | 1.14E-05 | 1.14E-05 |
| GOTERM_MF_FAT | GO:0003735~structural constituent of ribosome | 26 | 5.949657 | 1.12E-07 | UNIREF100_Q9FNP8, UNIREF100_Q9FI | 279 | 394 | 14806 | 3.501956 | 4.48E-05 | 4.48E-05 | 2.24E-05 |
| GOTERM_MF_FAT | GO:0043565~sequence-specific DNA binding | 19 | 4.347826 | 0.00456 | UNIREF100_O65683, UNIREF100_Q9LG | 279 | 483 | 14806 | 2.087565 | 0.840022 | 0.840022 | 0.367566 |
| GOTERM_MF_FAT | GO:0030145~manganese ion binding | 17 | 3.89016 | 2.91E-05 | UNIREF100_P48482, UNIREF100_Q9M9 | 279 | 257 | 14806 | 3.510341 | 0.011583 | 0.011583 | 0.003876 |
| GOTERM_MF_FAT | GO:0005525~GTP binding | 12 | 2.745995 | 0.011065 | UNIREF100_O82653, UNIREF100_Q389 | 279 | 263 | 14806 | 2.421358 | 0.988459 | 0.988459 | 0.590317 |
| GOTERM_MF_FAT | GO:0016209~antioxidant activity | 8 | 1.830664 | 0.017756 | UNIREF100_Q949U7, UNIREF100_Q9LY | 279 | 142 | 14806 | 2.989752 | 0.999241 | 0.999241 | 0.697998 |

**Supplementary Figure 1:** Analysis of miRNA nucleotide bias at each position. X-axis displays each position of miRNAs; Y-axis represents percent (%) of nucleotide. Purple bar represents uracil (U); green is guanine (G); red displays cytosine (C) and blue bars represent adenine (A).



**Supplementary Figure 2:** The GO annotation results of miRNA target genes in *B. napus*. Only the predicted target genes for miRNAs responding to chilling stress were considered.



# Chapter 3: Heterologous expression of *Brassica napus* pre-miR395f in *Arabidopsis thaliana* affects response to cold stress

## 3.1 Introduction

Plants being sessile in nature often encounter unfavourable environmental conditions, such as salinity, temperature extremes, drought and heavy metals. All these environmental abiotic stresses have devastating impact on plant growth, development, yield and biomass production (Zhang, 2015; Wani *et al.* 2016). As a result, plants have evolved intricate adaptive strategies at the morphological, cellular and physiological levels for sensing and responding to abiotic stresses (Krasensky and Jonal, 2012; Wani *et al.* 2016).

Cold stress (CS) is one of the commonly observed abiotic stresses, which causes significant economic losses to many agricultural crops (Sanghera *et al.* 2011; Zhang, 2015). Considerable progress has been made towards understanding and identifying CS-responsive genetic and associated signalling pathways (reviewed in Sanghera *et al.* 2011; Shi *et al.* 2015). A large number of cold-regulated (*COR*) genes encoding compatible solutes and protective proteins including dehydrins, heat shock proteins or transcription factors (TFs) such as APETALA2/ETHYLENE-RESPONSIVE FACTOR (AP2/ERF) have been identified (Sanghera *et al.* 2011; Shi *et al.* 2015, Megha *et al.* 2017). Furthermore, the Cold-responsive genes (*COR)* are known to be regulated by C-repeat Binding Factors (*CBFs*) and this regulatory network has been extensively studied in *Arabidopsis thaliana* (Thomashow, 1999). These *CBF*s regulate their downstream genes by binding to *cis*-acting elements designated as C-Repeat (CRT)/dehydration response element (DRE) in the promoter of *CORs* (Thomashow, 1999). In addition to the transcriptional network, these

genes are regulated by post-transcriptional regulators, such as miRNAs (Shrirram *et al.* 2016).

MicroRNAs (miRNAs) are a class of small RNAs (18-24 nt) that negatively regulate the expression of their target genes by cleavage or translational repression (Bartel, 2004; Voinnet, 2009; Li *et al.* 2013). While in animals, miRNAs regulate gene expression via sequence-specific targeting of messenger RNAs by the RNA-induced silencing complex (RISC), a perfect or near-perfect complementarity is required by the plant miRNAs to interact with their targets and direct target cleavage (Rhoades *et al.* 2002). The role of miRNAs as key post-transcriptional gene regulators in plant growth development is well established (De Lima *et al.* 2012; Li and Zhang 2016). Recent studies using high throughput sequencing and analysis have revealed the differential expression pattern of several miRNAs in response to various abiotic stresses including, cold, salt, drought and high temperature (Mittal *et al.* 2016; Wang *et al.* 2016; Shrirram *et al.* 2016). For example, miR167, miR168, miR171, miR319, miR393, miR394, miR395, miR396 and miR408 have been reported to be differentially expressed in different plant species under CS (Sunkar and Zhu, 2004; Theibut *et al.* 2012; Zhang *et al.* 2014; Karimi *et al.* 2016). Till date several miRNAs have been identified as functional regulators of CS by transgenic approaches (reviewed in Megha *et al.* 2017). For instance, in *A. thaliana* miR394-regulated cold tolerance was shown to be mediated through *CBF*-dependent cold responsive pathway (Song *et al.* 2016). Furthermore, the constitutive over-expression of miR396b in trifoliate orange enhanced cold tolerance by modulating the ethylene-polyamine homeostasis in transgenic plants (Zhang *et al.* 2016). These findings have opened up new avenues for

functional elucidation of miRNA-mediated gene regulation associated with CS at post-transcriptional level.

The *MIR395* gene family is evolutionary conserved across all major plant lineages (Guddeti *et al.* 2005; Zhang *et al.* 2006). In *A. thaliana* and *B. napus,* six members of miR395 family have been identified and designated as 'a-f' based on their genomic locations (Xie *et al.* 2005; Huang *et al.* 2010). Although lengths of precursor miRNA (pre-miRNAs) of miR395 members vary between *A. thaliana* and *B. napus*, the length (21 nt) and sequence of the mature miRNAs is conserved. Furthermore, the nucleotide sequences of miR395 a, d and e are identical to each other, as are of the members b, c and f (Kim *et al.* 2010). The targets of miR395 include *ATP sulfurylase* genes (*APS1*, *APS3* and *APS4*) and the high affinity sulfate transporter gene, *SULTR2;1* (Kawashima *et al.* 2009; Huang *et al.* 2010). Previous reports have shown that miR395 is an important component of sulphur assimilation pathway and its expression is induced differentially by sulphur starvation in *A. thaliana*, *B. napus* and *Oryza sativa* (Liang *et al.* 2012; Huang *et al.* 2010; Yuan *et al.* 2016; Li *et al.* 2017). *APS* catalyzes the first step in the sulfur assimilation while *SULTR2;1* is vital for its role in sulfate remobilization from mature to younger leaves (Liang *et al.* 2012; Huang *et al.* 2010). In addition, miR395 has been shown to be associated with response to abiotic stresses, such as low temperature, salt, drought, UV-exposure and nitrogen starvation (Ding *et al.* 2009; Kim *et al.* 2010; Liang *et al.* 2012; Wang *et al.* 2012; Wang *et al.* 2013; Kong *et al.* 2014; Megha *et al.* unpublished). In response to chilling stress, expression of miR395 has been found to be down-regulated in *Populus trichocarpa* and *P. tomentosa* (Lu *et al.* 2008; Chen *et al.* 2012). Transgenic

approaches to elucidate the function of miR395 in *A. thaliana* have found that miR395c/e over-expression results in differential seed germination under salt and drought stress (Kim *et al.* 2010). In the same study, under CS no significant differences were observed in seed germination and seedling growth between WT and miR395 overexpressing lines (Kim *et al.* 2010). Thus, experimental evidence supporting the functional role of miR395 in CS tolerance is still limiting.

In chapter 2 of this dissertation, four memebers of miR395 family (d, c, e, f) were identified after CS exposure of canola. Three members (c, e, f) were found to be DE based on $p \leq 0.01$ and fold change $\leq/\geq 1$. The expression of miR395c varied between different time points, whereas, miR395e and miR395f showed a trend. While miR395e was up-regulated over the course of 0 h-48 h after CS; miR395f expression showed a down-regulation after CS. We employed a transgenic approach, through heterologous over-expression of the *bna* pre-miR395f and pre-miR395e in *A. thaliana* to characterize their potential role in CS regulation. However, we were able to obtain only the pre-miR395f transgenic plants in a timely manner and hence proceeded with testing the *bna* pre-miR395f lines for their cold tolerance. Our results indicate that over-expression of pre-miR395f results in increased hypersensitivity towards cold. Changes in different physiological parameters such as malondialdheyde (MDA) content, electrolyte leakage and Guaiacol peroxidase (POD) activity were determined. This study also highlights the regulatory roles of miR395f and sulfur metabolism in response to CS.

## 3.2 Material and Methods

### 3.2.1 Plant material, growth conditions and abiotic stress treatments

In order to extract the genomic DNA for isolating the precursor of miR395f, leaf tissue was collected from three-week-old *B. napus* grown in soil. *A. thaliana* seeds were grown in soil (sunshine mix #4; SunGro) in a growth chamber at 22 °C with a 16 h photoperiod. For the survival experiments, three-week-old *A. thailana* plants were exposed to -5 °C for 2 h, transferred to 4 °C for 2 h and finally allowed to recover under normal conditions (22 °C with a 16 h photoperiod) and then photographed (Zhang *et al.* 2015). For CS assays, three-week-old plants grown on soil were transferred to 4 °C for 2 days. *A. thailana* ecotype Col-0 was used throughout the study.

### 3.2.2 Vector construction and plant transformation

A 400 bp fragment flanking the stem-loop fragment of *bna* pre-miR395f was amplified from the *B. napus* genome using primers listed in Supplementary File 1 and cloned downstream of Cauliflower Mosaic Virus 35S (CaMV35S) promoter of binary vector pBI121. The construct *pBI121: pre-miR395f* was sequenced to verify construct assembly. The construct, *pBI121: pre-miR395f* and empty vector *pBI121* were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and subsequently transformed into *A. thaliana* by floral dip protocol (Zhang *et al.* 2006). Kanamycin (50µg/ml) resistant plants regenerated from independent transformation events were further confirmed by genomic PCR using *NPTII* primers (Supplementary File 1). After further

selection of transgenic lines with a segregation ratio of 3:1, $T_4$ homozygous lines were used for further investigation.

### 3.2.3 Identification of homozygous *Arabidopsis* T-DNA insertion line

The miR395f-KO (*AT1G69797*) T-DNA insertion line (SALK_022530) was obtained from the *Arabidopsis* Biological Resource Centre. Genomic DNA was isolated from three-week-old rosette leaves of five individuals from SALK_022530 and WT control using Wizard® Genomic DNA Purification Kit (Promega) and screened using two combinations of primers, LB/RP and RP/RP, according to the protocol given at http://signal.salk.edu/tdnaprimers.2.html (Supplementary Figure 1)

### 3.2.4 Genomic DNA and total RNA extraction, quantitative real time PCR (qRT-PCR) analysis and stem-loop qRT-PCR

Genomic DNA from *B. napus* was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA). Total RNA was isolated from 100 mg plant samples using TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions and treated with RNAse-free-DNase (Promega, USA). Total RNA (1.5 µg) was used to synthesize first strand cDNA with Superscript II Reverse Transcriptase (Invitrogen, USA) according to manufacturer's instructions. Expression of mature miR395f in *A. thaliana* was assessed by stem-loop qRT PCR, as described by Varkonyi-Gasic and Hellens (2011) from total RNA and enriched small RNA (de Fátima Rosas-Cardenas *et al.* 2011). Expression of *bna* pre-miR395f, target transcripts and genes related to sulfur-metabolism was assessed by qRT-PCR using gene specific primers (Supplementary File 1). *UBC*

(Ubiquitin-conjugating) and *snoR101* (Small nucleolar RNA101) were used as endogenous genes for qRT-PCR and stem-loop qRT-PCR, respectively. All samples were analyzed in duplicate, for all three biological replicates. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to measure the transcript levels across the samples.

### 3.2.5 Determination of physiological parameters and Reactive Oxygen species (ROS) levels

Electrolyte leakage (EL), MDA content, chlorophyll content, carotenoid content and enzyme activity of POD were determined as described previously in Chapter 2. Accumulation of the two major types of ROS, $O_2^-$ and $H_2O_2$ was determined by histochemical staining. Staining of the leaves with Nitroblue Tetrazolium (NBT) and 3, 3'-Diaminobenzidine (DAB) was carried out as described previously (Kumar *et al.* 2014; Daudi and O' Brien, 2012).

### Statistical analysis

The data were statistically analysed by means of one-way analysis of variance (ANOVA) with post hoc comparisons using Dunnett t-test in SPSS, taking $P < 0.05$ as significant.

## 3.3 Results

### 3.3.1 Heterologous expression of *bna* pre-miR395f in *Arabidopsis thaliana*

In our previous study, we identified a total of 25 cold responsive miRNAs using next-generation sequencing (NGS) in *B. napus*, including miR395c, miR395e and miR395f (Megha *et al.* unpublished). In order to investigate the role of miR395f in mediating plant

responses to CS, a chimeric DNA construct containing the *bna* pre-miR395f sequence driven by CaMV35S promoter was generated (Figure 3.1a). This construct was then introduced into *A. thaliana* using *Agrobacterium*-mediated transformation and out of 12 independent transgenic events; three were selected after selection on Kanamycin for further analysis by PCR (Figure 3.1b). Higher expression of *bna* pre-miR395f was observed in all three lines (5 to 10 fold increase), as compared to the WT and VC by qRT-PCR analysis, suggesting that *bna* pre-miR395f was successfully expressed in *A. thaliana* (Figure 3.1c). After repeated attempts, we were not able to detect increased expression of mature miRNA395f in the transgenic lines (Figure 3.1d). We determined the expression of target genes under CS in WT *A. thaliana* plants. The expression of *APS1* and *APS3* transcripts decreased significantly, while the expression of *APS4* remained unchanged after CS in WT. The expression of *SULTR 2;1* transcript increased significantly after exposure of WT plants to CS (Figure 3.2). Furthermore, under control conditions, the transcript levels of all targets genes were lower in transgenics with a pattern opposite to that of miR395f (Figure 3.3). In addition, upon exposure to CS, the relative expression of *APS1* and *APS3* transcripts in transgenics showed a significant increase compared to WT and VC under CS, the expression of *APS4* and *SULTR 2;1* remained unchanged under CS in transgenics (Figure 3.3). This indicates that expression of miR395f target genes is differently regulated under CS in both WT and transgenic plants.

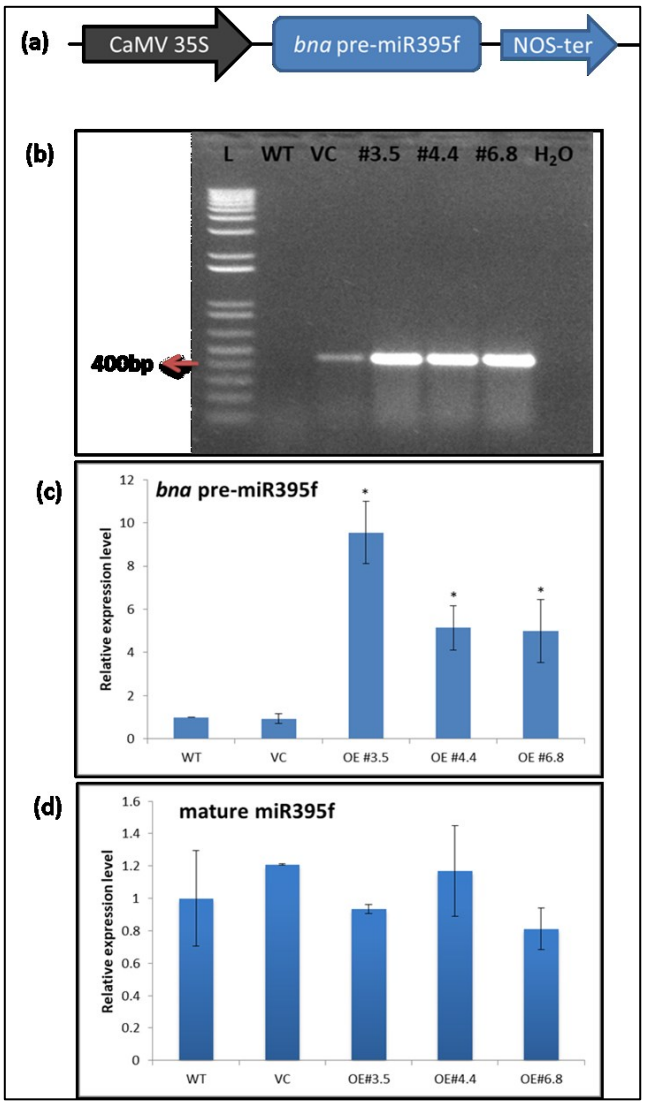



**Figure 3.1: Generation and characterization of transgenic *A. thaliana* plants over-expressing *bna* pre-miR395f.**

(a) Schematic diagram of the *bna* pre-miR395f over-expression construct under CaMV 35S promoter. NOS-ter, terminator of nopaline synthase gene. (b) Genomic PCR analysis of the putative transgenic plants using *NPTII* gene primers. L, 1 Kb ladder, $H_2O$ was used as negative control. (c) Expression of *bna* pre-miR395f in three transgenic lines, as determined by qRT-PCR. (d) Expression of maturemiR395f in three transgenic lines, as determined by Stem loop qRT-PCR. *UBC* (Ubiquitin-conjugating) and *snoR101* were used as endogenous control. Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic lines is significantly different from WT ($P$ <0.05).

### 3.3.2 Over-expression of the *bna* pre-miR395f results in altered sensitivity to freezing

To investigate the freezing tolerance of transgenic *A. thaliana* plants overexpressing *bna* pre-miR395f, we performed a whole plant survival test. Three-week-old plants of wild type (WT), vector control (VC), transgenic lines and miR395f-KO were subjected to -5 °C for 3 h, followed by 2 h recovery at 4 °C and survival percentage was determined after 2 days at 22 °C. Almost all of the transgenic rosette leaves were dead; however the vast majority of WT, VC and miR395f-KO regrew normally after recovery (Figure 3.4a). All three transgenic lines displayed a significantly ($P$ <0.05) lower survival rate (18.8-30.2%) when compared to a survival percentage of 44.8 %, 45.8 % and 43.8 % for WT, VC and miR395f-KO (Figure 3.4b).

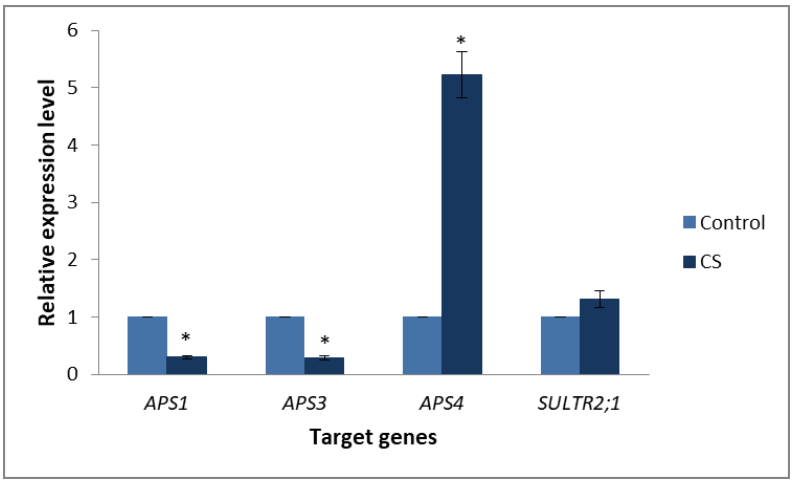



**Figure 3.2: Expression level of target mRNAs of miR395f in WT *A. thaliana* under control and CS conditions as determined by stem loop qRT-PCR.**

*UBC* (Ubiquitin-conjugating) was used as endogenous control. Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic lines is significantly different from WT ($P$ <0.05).

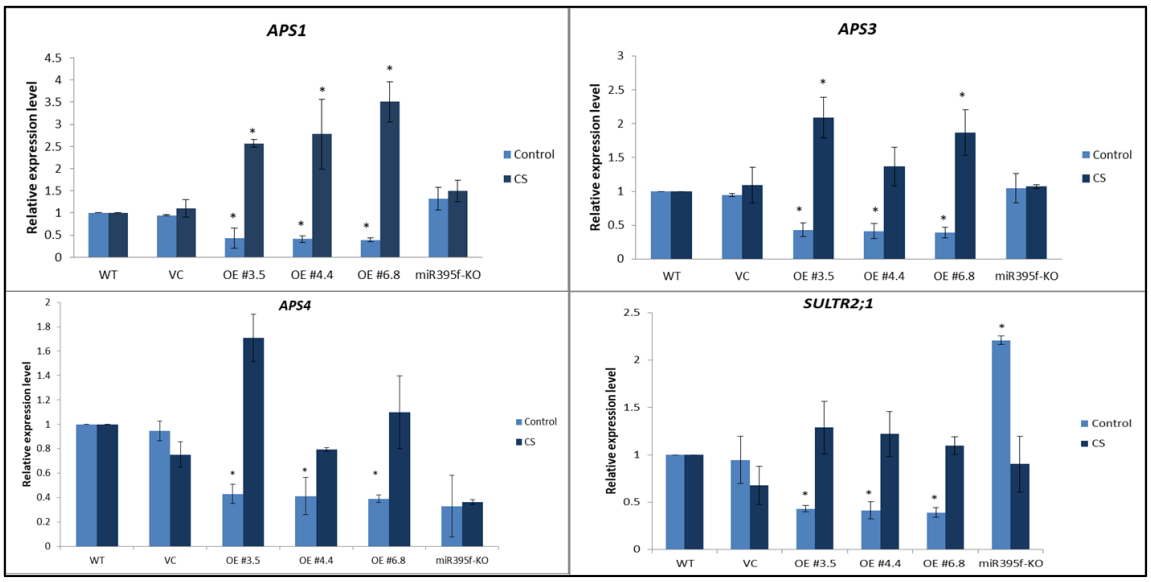



**Figure 3.3: Expression level of target mRNAs of miR395f during control and CS conditions as determined by qRT-PCR.**

*UBC* (Ubiquitin-conjugating) was used as endogenous control. Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic lines is significantly different from WT ($P$ <0.05).

We further carried out an electrolyte leakage (EL) test to determine the extent of freezing injury of the membranes of transgenic plants. Similar levels of EL were observed in the miR395f-KO (38 %) and WT (37.9 %) following exposure to freezing temperature. The transgenic lines exposed to freezing stress exhibited an enhanced EL (56.8 - 68.6 %), much higher than that measured in WT (37.9 %) and VC (35.1 %) (Figure 3.4c). These results indicate that transgenic *A. thaliana* with heterologous over-expression of *bna* pre-miR395f displayed increased freezing sensitivity, compared to WT, VC and miR395f-KO plants.

### 3.3.3 Changes in physiological parameters in transgenic plants under CS conditions

The hypersensitivity of transgenic plants to freezing stress prompted us to investigate whether there were physiological differences between WT and transgenic plants that could explain the altered sensitivity to freezing temperatures. Determination of MDA concentration is widely used to monitor increased lipid peroxidation resulting from CS (Taulavouri *et al.* 2001). Under control conditions, there was no significant difference in the level of MDA in WT, VC, transgenic lines and miR395f-KO, except for OE #3.5 (Figure 3.5a).

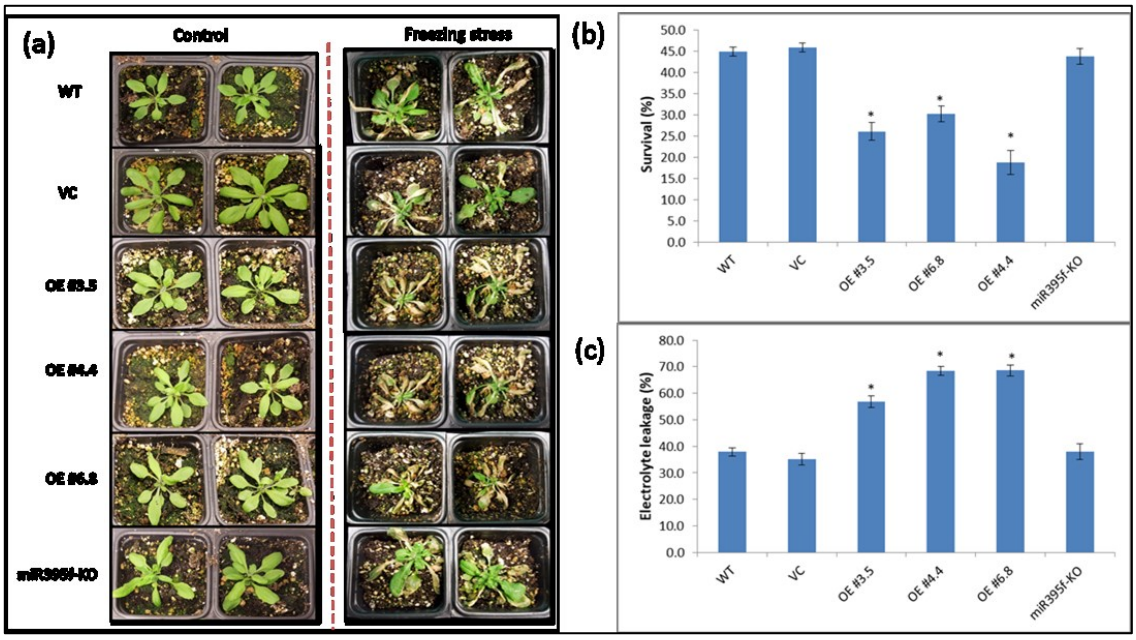



**Figure 3.4: Stress tolerance of WT, VC, OE#3.5, OE#4.4, OE#6.8 and miR395f-KO subjected to freezing stress. (a) Performance of lines exposed to -5 °C for 2 h, followed by 2 days of recovery at 22 °C. (b) The survival percentage determined after 2 days of recovery (c) Percent electrolyte leakage determined after 2 days of recovery.**

Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic lines is significantly different from WT ($P$ <0.05).

In the transgenic lines, the level of MDA was significantly ($P$ <0.05) higher after exposure to CS for 48 h as compared to WT plants, suggesting that the over-expression lines suffered more severe membrane damage (Figure 3.5a). Furthermore, reduction in Chl-a, Chl-b and carotenoid levels were determined under CS conditions. Except a significant ($P$ <0.05) increase in carotenoid level in miR395f-KO, no significant changes in Chl-a, Chl-b and carotenoids were observed among all the lines tested after CS (Figure 3.5b).

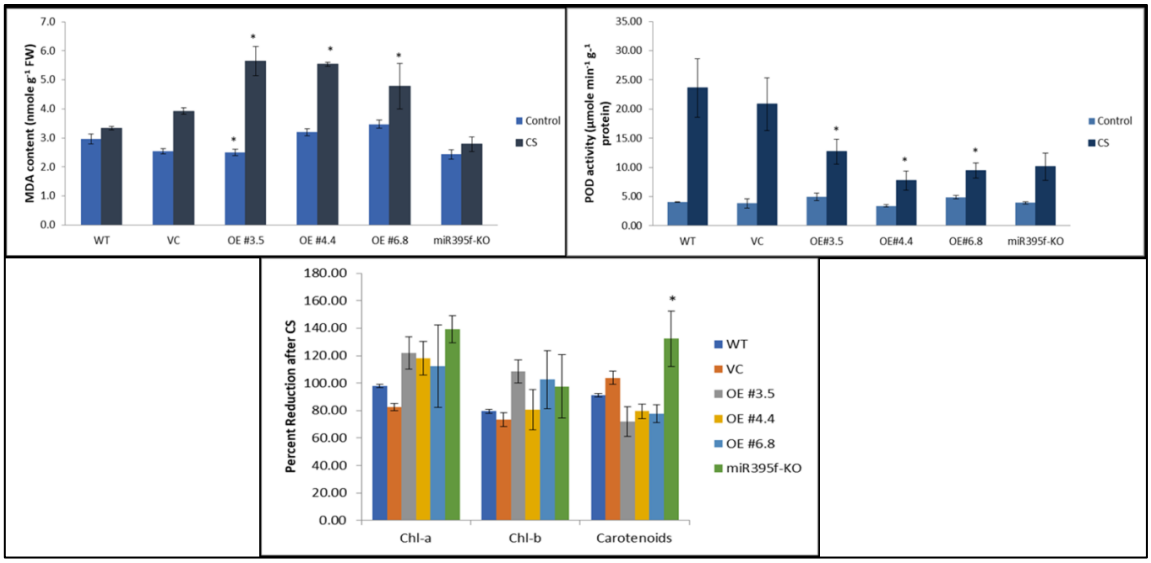



**Figure 3.5: Changes in (a) malondialdheyde (MDA), (b) Guaiacol peroxidase (POD) activity and, (c) percentage reduction of Chl-a, Chl-b and carotenoids under control and CS conditions in WT, VC, OE#3.5, OE#4.4, OE#6.8 and miR395f-KO.**

Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic lines is significantly different from WT ($P$ <0.05).

We also determined the enzymatic activity of POD in both control and plants subjected to CS at 4 °C. No significant change in enzymatic activity of POD was observed for all the

lines growing under control conditions. After CS, all the transgenic lines showed a significant ($P$ <0.05) decrease of 1.6-2.7 fold in POD activity when compared to WT and VC (Figure 3.5c). Although the miR395f-KO showed similar levels of survival percentage, EL and MDA content, the POD activity of miR395f-KO was two-fold less compared to WT and VC (Figure 3.5c), indicating that regulation of CS in miR395f-KO might be through a different unknown mechanism.

Sulfur-containing compounds, such as, glutathione, are known to modulate ROS levels, thus playing a role in stress tolerance. We therefore compared ROS levels in the WT, VC, transgenic plants and miR395f-KO before and after exposure to CS using histochemical staining. Without CS, the leaves of transgenic, WT, VC and miR395f-KO were equivalently stained indicating that accumulation of ROS was similar in these lines (Figure 3.6). However, upon exposure to CS, the leaves of transgenic lines were stained heavily by both NBT and DAB than those of the WT, VC and miR395f-KO (Figure 3.6). In the present study, we observed a lower level of POD activity and a higher accumulation of ROS in transgenic lines under CS. Thus over-expression of *bna* pre-miR395f in *A. thaliana* can in part be co-related with enhanced ROS levels and decreased scavenging capacity of POD thereby, increasing sensitivity to CS.

### 3.3.4 Changes in expression of transcript levels of enzymes related to sulfur-metabolism and antioxidant machinery

As alluded to several times previously, miR395 is known to mediate sulphur homeostasis by regulating its accumulation and allocation in plant species such as *A. thaliana*, *O. sativa* and *B. napus*. Thus in the present study, we measured the changes in

transcript levels of the rate limiting enzymes of sulphur-containing compounds and amino acids, such as, glutathione, methionine and cysteine under control and CS conditions. Under control conditions, no significant differences were observed in the expression of Glutamate-cysteine ligase *(GSH1),* two isoforms of Cystathionine-γ-synthase *(CGS1* and *CGS2),* Serine acetyl-transferase *(Serate 3;1* and *Serate 3;2)* and O-acetylserine (thiol) lyase *(OAS-TL)* isoform A1 *(OASA1)* transcripts between transgenic lines and WT (Figure 3.7a). Under CS, expression of *GSH1, CGS1, Serate 3;1* and *OASA1* increased significantly in transgenic lines when compared to WT and VC (Figure 3.7a). For instance, after exposure to CS, the expression levels of *GSH1, CGS1, Serate 3;1* and *OASA1* in transgenic lines were 1.7-10.5 fold higher than in WT and VC (Figure 3.7a). The expression of *CGS2* showed no significant change in its expression in transgenics compared to WT under control and CS conditions, while the expression of *Serate 3;2* transcript in transgenics decreased significantly (~29 fold) under control conditions compared to WT (Figure 3.7a). These results indicated that CS altered the expression profile of the rate limiting enzymes of sulphur-containing compounds and antioxidant enzymes in miR395f OE lines. In order to further determine whether changes in genes encoding antioxidant enzymes requiring oxidation / reduction of sulfur in their reaction mechanisms could contribute to the increased sensitivity of miR395f transgenic plants, we also monitored the changes in transcript levels for Glutathione reductase (*GR1, GR2)*, NADPH-dependent thioredoxin reductase (*NTRA, NTRB and NTRC)* and Glutathione peroxidase (*GPX 1-7*). Under CS, the expression of *GR1* transcript showed an increase of~2.5 fold in transgenic lines compared with WT (Figure 3.7b).

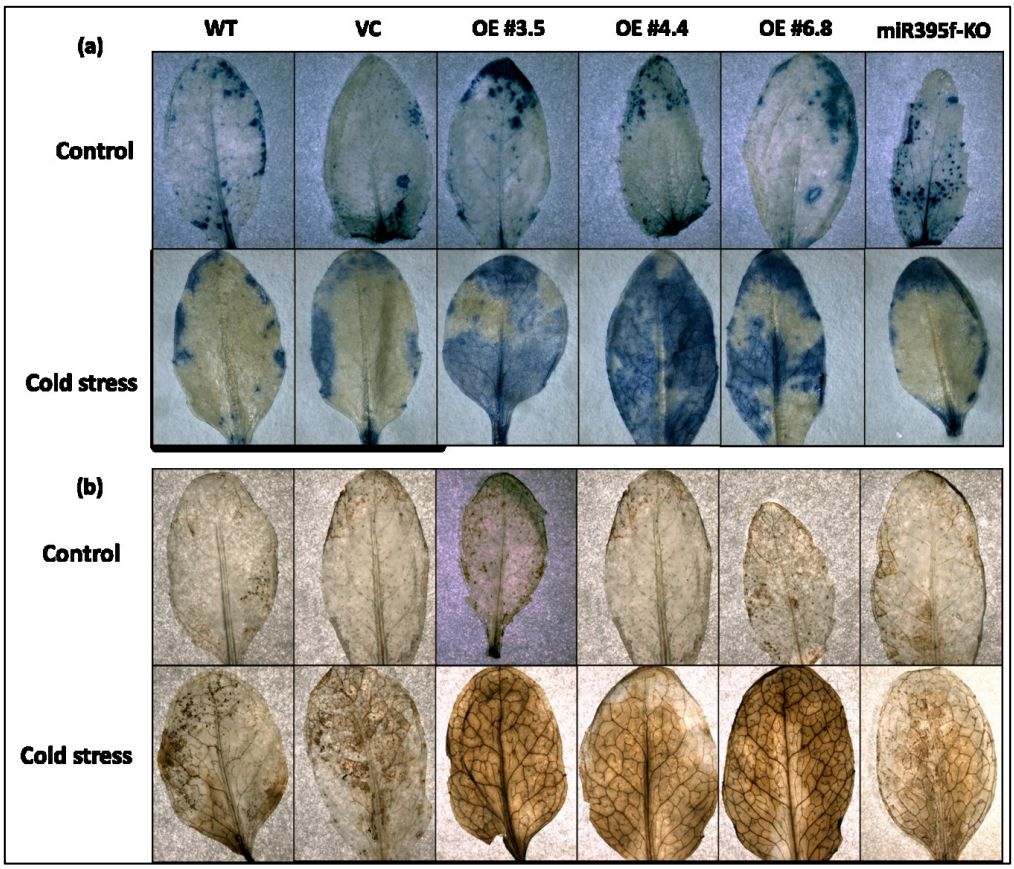



**Figure 3.6: Analysis of ROS accumulation before and after CS. (a and b) *In situ* accumulation of $O_2^-$ and $H_2O_2$ in the WT, VC, OE#3.5, OE#4.4, OE#6.8 and miR395f-KO with and without CS, as revealed by NBT (a) and DAB (b) staining, respectively**.

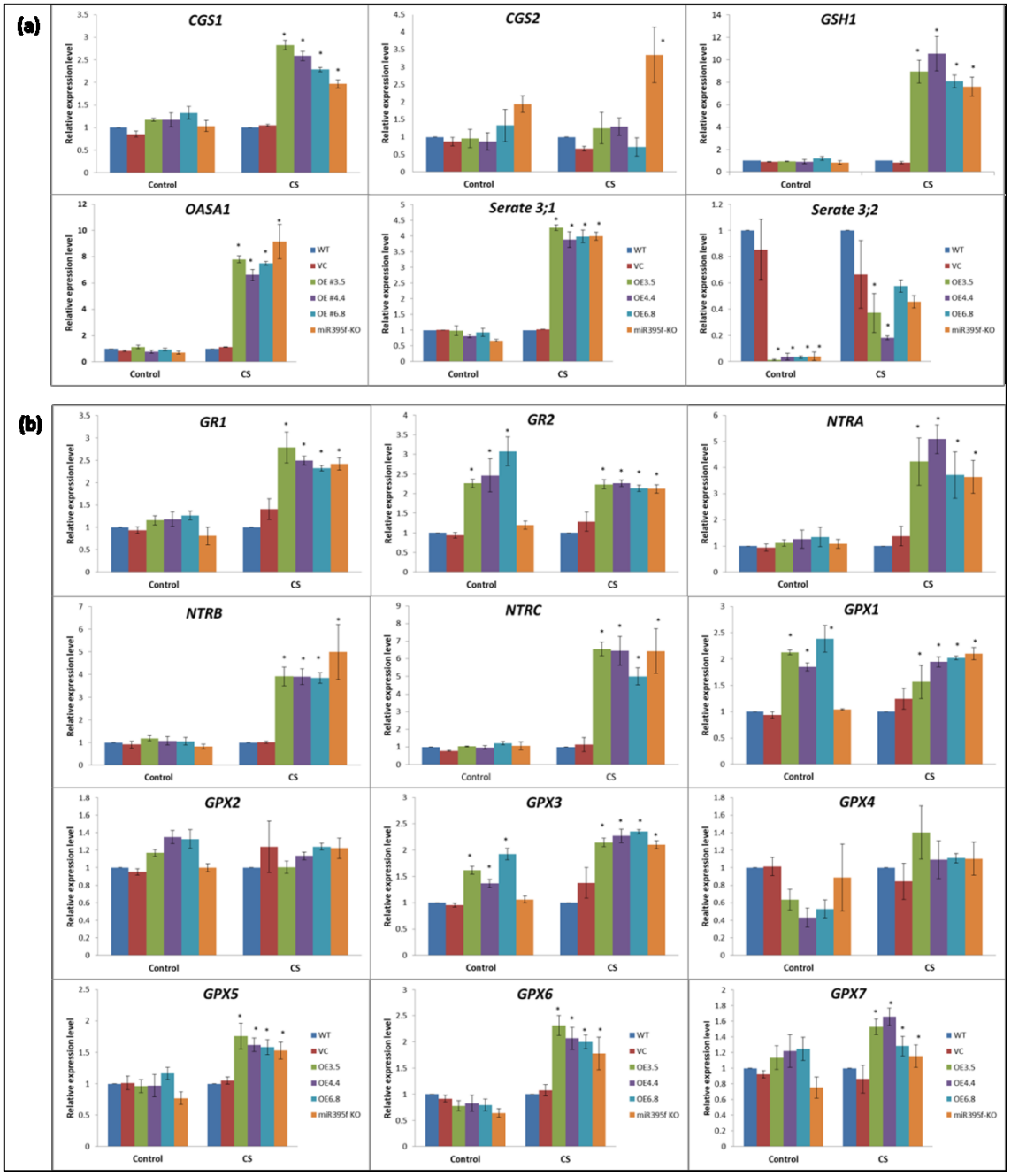



**Figure 3.7: Changes in expression level of transcripts related to sulfur metabolism (a) and antioxidant enzymes (b) in plants grown under control and CS conditions.**

Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic lines is significantly different from WT ($P$ <0.05).

On the other hand, relative expression of *GR2* transcript in transgenics under control conditions was significantly higher than control WT and VC (Figure 3.7b). Although, in comparison to WT (CS), transgenic lines showed an increase of ~2.2 fold in the expression of *GR2* transcripts, a decrease in expression (0.69-1 fold) was observed when transgenic lines grown under control and CS conditions were compared suggesting that CS affects the expression of *GR2* transcripts. While, the expression level of *NTRA, NTRB,* and *NTRC* showed no significant change in control transgenic plants with respect to WT control; their expression increased significantly after CS in transgenics (Figure 3.7b). Furthermore, the expression of three of Glutathione peroxidase transcripts *i.e., GPX2, GPX4* and *GPX7* remain unchanged under control and CS conditions for all lines tested (Figure 3.7b), except for *GPX4* whose expression was reduced in transgenics compared to WT control. The expression level of *GPX1, GPX3, GPX5* and *GPX6* transcripts showed a significant yet modest increase of 1.5–2.0 fold after CS in transgenics in comparison to WT (CS) (except *GPX2)* (Figure 3.7b). The expression level of all these transcripts when determined in miR395f-KO was increased after CS compared to WT under CS (Figure 3.7b). These results indicate that heterologous over-expression of *bna* pre-miR395f in *A. thaliana* causes changes in expression profile of genes involved in sulfur metabolism and redox homeostasis.

**3.4 Discussion**

Over the recent years, a large number of miRNAs have been identified and characterized for their role in regulation of normal plant growth, development and response towards cold and other environmental stresses (Fernandez *et al.* 2014; Suzuki *et al.* 2014).

The biological functions of miR395 have been investigated mainly in *A. thaliana* by the manipulation of various members of this family (Kawashima *et al.* 2009; Liang *et al*. 2010; Kawashima *et al.* 2011; Ai *et al.* 2016). The initial studies reported expression of miR395 to be highly induced under sulfate limiting conditions in *A. thaliana* (Jones-Rhoades and Bartel, 2004; Kawashima *et al.* 2009). Furthermore, over-expression of miR395d and f showed an over-accumulation of sulfate in transgenic *A. thaliana* and suppression of their target transcripts (Liang *et al.* 2010; Ai *et al.* 2016). miR395f OE plants showed sulfate starvation *i.e.* slight chlorosis in their leaves and reduced growth compared to WT plants (Liang *et al.* 2010). In spite of a higher sulfate content in miR395f OE plants, the display of sulfur deficient symptoms indicate that sulfur assimilation may be repressed in these plants (Liang *et al.* 2010). The expression of miR395f was also induced in response to sulfate limitation in the leaves of *B. napus* and rice (Huang *et al.* 2010, Yuan *et al.* 2016) and the heterologous expression of rice *pri-miR395h* in tobacco retarded the plant growth (Yuan *et al*. 2016). These studies point to the critical role of miR395 family in regulation of sulfate accumulation and allocation. However, even though the expression of various members of miR395 family has been revealed to be differentially regulated in response to CS (Sunkar and Zhu 2004; Zhang *et al.* 2009; Chen *et al.* 2012; Cao *et al.* 2015), there is no convincing evidence for the role of miR395 in CS regulation.

In the current study, heterologous expression of *B. napus* pre-miR395f in *A. thaliana* was used to understand its role in CS regulation. Although we observed an increase in expression of *bna* pre-miR395f in transgenic lines compared to WT, no increase for mature miR395f was observed in transgenics. The active mature miRNA is generally

considered to negatively regulate gene expression but recent evidence suggests that pri-/ pre- miRNAs have direct functions in regulating gene expression (Trujillo *et al.* 2010; Yue *et al.* 2011; Roy-Chaudhuri *et al.* 2014; Zhu *et al.* 2015). It has been shown that pri-let-7 can directly interact and repress target expression in the presence of truncated and non-functional mature let-7 (Yue *et al.* 2011). In addition, Kay's group (Roy-Chaudhuri *et al.* 2014) reported that pri-/pre-miR151 directly regulates the expression of *Ef26* transcript by binding to its 3'-untranslated region (3'-UTR) thereby supporting the hypothesis that miRNA precursors are not mere biogenesis intermediates but can also act as direct regulators of miRNA activity . At this time, to our knowledge, there are no reports of pri-/ pre-miRNAs regulating the expression of target genes in plants. It can be speculated that the increased sensitivity of plants over-expressing *bna* pre-miR395f can be attributed to regulation of target genes by pre-miR395f rather than mature miR395f. miRNAs usually negatively regulate the expression of their target genes, and the expression of *APS1, APS3, APS4* and *SULTR2;1* transcripts was suppressed in transgenic lines grown under control conditions, but on exposure to CS expression level of *APS1*, *APS3* increased while that of *APS4* and *SULTR2;1* transcripts in transgenics was comparable to WT (Figure 3.2). Thus, from the differential expression of miR395f targets under CS, it might be speculated that the transcript levels of the target genes are regulated by another, as of yet unknown, mechanism in addition to suppression by miR395.

We observed symptoms of reduced growth and slight chlorosis in leaves of plants over-expressing *bna* pre-miR395f under control conditions (Figure 3.4a). Furthermore, transgenic *A. thaliana* lines overexpressing *bna* pre-miR395f exhibited enhanced sensitivity

to both freezing (-5 °C) and CS (4 °C). In addition, an increased EL was observed in transgenic lines, indicating greater membrane damage compared to WT. It has been demonstrated that CS causes the elevated levels of ROS which can be very lethal and can cause damage to proteins, DNA and lipids thereby affecting the normal cellular functioning (Foyer and Noctor, 2005). Increased levels of the histochemical staining of transgenic lines after exposure to CS as compared to the WT, indicates higher accumulation of $H_2O_2$ and $O_2^-$. Excess $H_2O_2$ can be transferred via the Haber-Weiss reaction to form highly reactive oxidant hydroxyl radical (OH•) which leads to the lipid peroxidation which increases the membrane fluidity causing the membrane to be leaky (Das and Roychoudhury, 2014). In the present study, MDA content which was found to be increased in stressed plants indicated severe lipid peroxidation. Also, the increase in $H_2O_2$ levels in transgenic lines was further confirmed by a decrease in the $H_2O_2$ scavenging activity of antioxidant enzyme POD. Thus, the hypersensitive response of transgenic plants to both freezing and CS can be attributed to both over-expression of *bna* pre-miR395f and reduced growth of plants.

The expression of transgene driven by 35S promoter has been reported to be influenced by changes in growth conditions such as temperature and day length. Although there are no reports of increased expression of transgene driven by 35S promoter under CS conditions, reduced expression has been reported previously after CS. For instance exposure of tobacco plants to 4°C resulted in more than 80% decrease in the transgene expression (Schnurr and Guerra, 2000). Similarly, in *A. thaliana* plants grown for three weeks at 4°C, the 35S-driven transgene expression dropped significantly (Bokyo *et al.* 2005). Thus, it can be speculated that expression of 35S promoter was affected by CS in

transgenic plants which further affected the expression of *bna* pre-miR395f in transgenic *A. thaliana.*

In order to establish a link between enhanced sensitivity to CS conferred by miR395f OE, sulphur metabolism and components of ROS scavenging machinery, we determined the transcript levels of few rate limiting enzymes in sulfur assimilation pathway, such as, *GSH1* (glutathione biosynthesis)*, CGS1* and *CGS2* (synthesis of methionine from cysteine)*, Serate 3:1* and *OASA1* (involved in cysteine synthesis) and of some antioxidant enzymes. Although, the miR395f OE plants exhibited a sensitive phenotype in response to freezing and CS exposure, the expression level of 77.7 % of the transcripts (14 out of 18) was found to increase in response to CS. It can be speculated that although there was a higher expression of antioxidant genes after CS, this increase was not enough to scavenge the ROS produced which resulted in hypersensitivity response of transgenics after CS. In addition, it is also known that expression levels of mRNA and protein often show a correlation of 40 % (de Sousa Abreu *et al*. 2009; Maier *et al.* 2009). This discrepancy is often attributed to transcriptional, post-transcriptional (RNA processing, RNA stability) or translational regulation (Kawaguchi *et al.* 2004). Thus, a poor correlation between protein and mRNA level of antioxidant enzymes measured in this study can be used to explain the hypersensitive response of transgenics. Whether or how transcripts of other antioxidant enzymes, apart from the ones tested, were influenced by *bna* pre-miR395f OE in the transgenic plants remains to be determined.

In conclusion, our findings demonstrate that pre-miR395f has a negative impact on plant response to CS and thus provide a valuable basis to further investigate the role and molecular mechanism of miR395f mediated regulation of CS responses.

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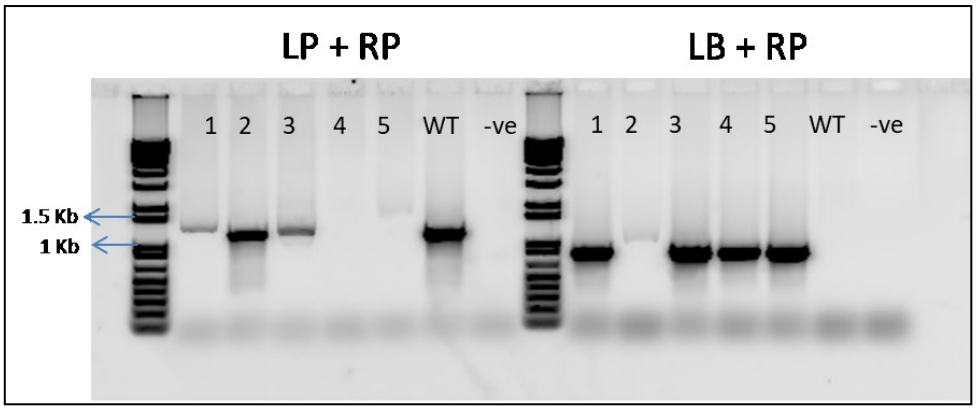
## Supplementary Files

**Supplemntary File 1**: List of primers used in this study.

| | |
|---|---|
| 395f_R.1_Sac1 | GAC TAG GAG CTC TGAAGATGCACATAACTCACCTG |
| 395f_F.2_BamH1 | GAC AGT GGA TCC CCA TCC CTA AGA TAT CCC ATT GT |
| LP_miRNA395f | CGGGAGAGGAATACGGTTTAG |
| RP_miRNA395f | CGTTAAAGGCCATGTTTAGGG |
| LB | TGGTTCACGTAGTGGGCCATCG |
| q395f_F | CGCACAATCCCACTATCCTT |
| q395f_R | TGCGAAACCGCTTGATAAAT |
| RT_miR395f* | GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC CTGAAG |
| F_miR395f* | CGC CTA ATG gagtccccccaaaca |
| UNIVERSAL REVE | GTGCAGGGTCCGAGGT |
| Kanamycin_F | TTCTTTTTGTCAAGACCGACCT |
| Kanamycin_R | CACAGTCGATGAATCCAGAAAA |
| NTRA_F | CTAGCCACCGCGTTTTCTTC |
| NTRA_R | TAGATCGCCGCCGTGTGT |
| NTRB_F | CCACCGACGTCGAGAATTTC |
| NTRB_R | GACTTTCGTCACCGTCTCTGTAAA |
| NTRC_F | TTCAGGAGGCGAGATTATCGA |
| NTRC_R | CGCCCATCTGATACCCTTCA |
| GR1_F | TCCTGGACATGAGCTGGCTAT |
| GR1_R | CCATTCCACGCCATATTGATG |
| GR2_F | GTCGCAAGCCCAACACAAA |
| GR2_R | ATCCCCAACAGCCCAGATG |
| GPX1_F | CTTTTCCCTGCAATCAGTTTGG |
| GPX1_R | GCTTGGTCCATTCACGTCAA |
| GPX2_F | TGGCGGATGAATCTCCAAAG |
| GPX2_R | ACCACATTTGGAAGCAACGTT |
| GPX3_F | CAAGGAAATGTCGTTGACCGATA |
| GPX3_R | TTGATGCGATGCTTTTTGCT |
| GPX4_F | GATCTTGGCATTCCCTTGCA |
| GPX4_R | CGTTTACGCGTACCTTTTGGA |
| GPX5_F | TTTTGTGGTATTGGCGTTTCCT |
| GPX5_R | CACGCACCTTTTGGAAAACA |
| GPX6_F | CTGCTTCTTCCGAACCCAAA |
| GPX6_R | GCCACATTGAGAAGCAACGTT |
| GPX7_F | TTGCAATCAATTTGGAGGTCAA |
| GPX7_R | GAGCTGTGCTTGGTCCATTCA |
| GSH1_F | TTCCCTGTCTCCCTGGTGAA |
| GSH1_R | CCTCCAGGGACCTCCATCA |
| Serat3;1_F | TGCATTGCAAAGCCGAATAA |
| Serat3;1_R | TGCCTATCACAGCGGTCTCA |
| Serat3;2_F | AGTTCACGTCCACTTCGTGTGT |
| Serat3;2_F | GCGTGTGACCGTGATTCAGT |
| OASA1_F | CGGAAGAGATTTTGGCGAAA |
| OASA1_R | GATTTTGCCACCAGTGCCTTT |
| CGS1_F | AGCGTCGATGAGGAGGTTGT |
| CGS1_R | TCACCGGCATGAACAGTGA |
| CGS2_F | TCCGGTTCAATGGAGTTGGT |
| CGS2_R | TGTCTTACACGAAGATGCATCGT |
| SnoR101_F | GGGATACACTTGATCTCTGAACT |
| SnoR101_R | GCATCAGCAGACCAGTAGTTATC |
| UBC_F | TGCTTGGAGTCCTGCTTGGA |
| UBC_R | TGTGCCATTGAATTGAACCCTCT |
| APS1_F | TGCAATGGATGCATGTATTAAGC |
| APS1_R | ATAGGCACGGACATGTTAACGA |
| APS3_F | GGATTTGCCGAGAGTGAGATTG |
| APS3_R | GACCCATCATCGAGATTCAACA |
| APS4_F | CAAGCTGAACCGTGTGGATCT |
| APS4_R | CGAGCCGGAACGAGTTAAAA |

**Supplementary Figure 1**: Genotyping of T-DNA insertion line (SALK_022530) using two sets of primers; gene specific LP and RP; and LB (Left border primer of the T-DNA insertion) and RP. Plant # 4 and 5 showed amplification with only LB and gene specific RP indicating the presence of both copies of mutated allele and hence were used for further analysis.



## Chapter 4: Potential miRNA-SSR markers for use in *Brassica napus* breeding and research

### 4.1 Introduction

The family of *Brassicaceae* includes six cultivated species: *B. rapa* (AA, 2*n* = 20), *B. nigra* (BB, 2*n* = 16), *B. olearacea* (CC, 2*n* = 18), *B. juncea* (AABB, 2*n* = 36), *B. carinata* (BBCC, 2*n* = 34) and *B. napus* (AACC, 2*n* = 38) (U, 1935). The genetic relationship of these six species is described as U's triangle in which the three allotetraploid species evolved from interspecific hybridization between the three diploid species (U, 1935). Various types of molecular markers including restriction fragment length polymorphism (RFLP) (Sebastian *et al.* 2000), rapid amplification of polymorphic DNA (RAPD) (Khan *et al.* 2008), amplified fragment length polymorphism (AFLP) (Li *et al.* 2011) and simple sequence repeats (SSRs) (Hobson and Rahman, 2016) have been used to assess the genetic diversity in *Brassica* for use of this information in breeding.

Simple sequence repeats (often defined as 1–6 bp) have been detected ubiquitously in genomic regions of all eukaryotic organisms (Tautz and Renz, 1984) and remain the marker of choice for genome mapping, evolutionary and population studies owing to their co-dominant nature, high reproducibility, high variability and dense distribution throughout the genome (Hobson and Rahman, 2016). SSRs have been previously used for assessment of genetic diversity among different accessions of *B. napus* (Bus *et al*. 2011; Gyawali *et al.* 2013) and *B. rapa* (Annisa *et al.* 2013; Hobson and Rahman, 2016). Furthermore, the functional role of SSRs had also been corroborated in different plants species in response to developmental changes and abiotic stress exposure. For instance, several QTLs have been identified by use of SSRs for kernel size and milling quality in wheat (Breseghello and

Sorrells, 2006), for salt and waterlogging tolerance in barley (Zhou *et al.* 2012) and rice (Alam *et al.* 2011), submergence tolerance in perennial ryegrass (Yu *et al.* 2011) and soybean (Hamwieh *et al.* 2011); for seed coat color, oil content and seed glucosinolate content (Hasan *et al.* 2008; Qu *et al.* 2015) and disease resistance (Hasan and Rahman 2016; Fredua-Agyeman and Rahman 2016) in *B. napus* and for cold tolerance in *B. rapa* (Huang *et al.* 2017).

Low temperature (LT) stress is a major abiotic stress and causes tissue injury, including wilting of leaves, necrosis of tissues and chlorosis, which significantly limits the productivity of crops (Mahajan and Tuteja, 2005; Sanghera *et al.* 2011). LT stress can be classified as CS (< 10 °C) and freezing stress (< 0°C), based on the temperature affecting certain plant types (Levitt, 1980). Cold stress causes injury to plants when exposed to low but non-freezing temperatures (Levitt, 1980). LT survival is a complex trait that involves vernalization response, cold-acclimation and freezing tolerance (Rife and Zeinali, 2003). Research has shown that as normal cellular functions are disrupted during CS; plants undergo remodeling of cell structures and reprogramming of gene expression to minimize cold damage (Mahajan and Tuteja, 2005; Sanghera *et al.* 2011). The reprogramming of gene expression in response to LT occurs at transcriptional, post-transcriptional and translational level (Chinnusamy *et al.* 2007; Jeknić *et al.* 2014). Among the different regulatory elements, small endogenous non-coding RNAs, microRNAs (miRNAs), are known to play significant roles in post-transcriptional processes by directing cleavage or translational repression in plants (Jones-Rhoades *et al.* 2006; Groszhans and Filipowicz, 2008; Moran *et al.* 2017; Megha *et al.* 2017). MiRNAs have been demonstrated to be involved in the regulation of CS for the first time by Sunkar and Zhu (2004) in *A. thaliana*

and since then various studies in different plant species have confirmed the role of miRNAs in response to CS (Chen *et al.* 2012; Zhang *et al.* 2014; Cao *et al.* 2015; Sun *et al*. 2015; Karimi *et al.* 2016).

*Brassica* species comprise an exceptionally diverse group of crops providing vegetables, condiments and oil and thus play an important role in agriculture. A recent study from our lab has generated a well annotated miRNAome for CS responses in spring *B. napus* and provided evidence for the involvement of miRNA-target gene regulatory networks in mediating responses to CS (Megha *et al.*, unpublished). In addition, differential patterns of miRNA and their target gene expression were observed in spring and winter *B. napus* when subjected to CS (Megha *et al*., unpublished). Spring type *B. napus* has lower frost tolerance when compared to winter types owing to their limited ability to undergo proper hardening that involves growth cessation and maintenance of high photosynthetic activity during cold acclimation (Rapacz and Janowiak, 1998). In other words, temperate winter type oilseed rape plants exhibit freezing tolerance and this ability is further enhanced through cold-acclimation (Rapazc and Janowiak, 1998). Seeding of spring *B. napus* canola in Canada is delayed to early- to mid-May to avoid frost damage to the seedling. Delayed seeding often exposes the crop to summer heat at the flowering stage which can result in abnormal flower and silique set and thus reduce seed yield (Angadi *et al.* 2000). It would therefore, be extremely useful to identify functional markers that can differentiate between spring and winter types and derivatives of their crosses to develop markers for the development of a frost tolerant spring *B. napus* cultivar. Although, Huang and co-workers identified three SSR markers, which can distinguish between cold-resistant

and susceptible cultivars of *B. rapa* (Huang *et al.* 2017), there are no other reports of cold-specific SSRs/miR-SSRs in *B. napus*.

In this study, we have mined SSRs in miRNA genes of *B. napus* and used them to perform an analysis of genetic diversity among spring and winter growth habit type of *B. napus* and *B. rapa*, and recombinant inbred lines (RILs) derived from cross between these two types of *B. napus*. Our results are presented and discussed within the context of information related to cold related miR-SSR markers as well. To our knowledge, this is the first report describing the potential utility of miRNA-based SSR markers to classify *Brassica* lines with differential responses to CS.

## 4.2 Material and Methods

### 4.2.1 Plant materials and DNA extraction

In the present study, a total of 64 *Brassica* accessions were used: 20 lines of spring *B. napus*, 14 of winter *B. napus*, 10 of winter *B. rapa* and 20 RILs derived from winter × spring *B. napus* crosses (Table 4.1). Genomic DNA was extracted from 100 mg of new leaf tissue from representative individuals using Wizard® Genomic DNA Purification Kit (Promega). Isolated DNA was quantified using Nanodrop 1000 (Thermo Scientific) and diluted to a final concentration of 25 ng $\mu L^{-1}$ in nuclease free water.

### 4.2.2 Mining of SSR markers from *B. napus* miRNA genes

Precursor-miRNA (pre-miRNA) sequences (90 sequences) from *B. napus* genome were downloaded from miRBase (release 21) (Kozomara and Griffiths-Jones, 2014) and were

**Table 4.1**: **Details of the *Brassica* oilseed cultivars/lines used in this study**.

| Scientific name | Cultivar/Line name | Origin[1] | Scientific name | Cultivar/Line name | Origin[1] |
|---|---|---|---|---|---|
| ***Panel 1 (Spring type)*** | | | ***Panel 2 (Winter type)*** | | |
| *B. napus* | Altex-1 | Canada | *B. napus* | Ibiza | Europe |
| *B. napus* | Alto | Canada | *B. napus* | Diffusion | Europe |
| *B. napus* | Peace | Canada | *B. napus* | Vision | Europe |
| *B. napus* | Quantum | Canada | *B. napus* | Galilieo | Europe |
| *B. napus* | Q2 | Canada | *B. napus* | Goya | Europe |
| *B. napus* | Hi-Q | Canada | *B. napus* | Cult | Europe |
| *B. napus* | SILEX | Canada | *B. napus* | Da Vinci | Europe |
| *B. napus* | Roper | Canada | *B. napus* | Billy | Europe |
| *B. napus* | A04-73NA | Canada | *B. napus* | Lorenz | Europe |
| *B. napus* | A07-28NA | Canada | *B. napus* | Exocet | Europe |
| *B. napus* | Conquest | Canada | *B. napus* | Exagone | Europe |
| *B. napus* | A99-13NR | Canada | *B. napus* | Aviso | Europe |
| *B. napus* | A03-3NR | Canada | *B. napus* | Verona | Europe |
| *B. napus* | A06-9NR | Canada | *B. napus* | Favorite | Europe |
| *B. napus* | A07-26NR | Canada | ***Panel 4 (Spring type RILs from W × S[2])*** | | |
| *B. napus* | Cougar | Canada | *B. napus* | A07-38NR | Canada |
| *B. napus* | A05-4NI | Canada | *B. napus* | A07-45NR | Canada |
| *B. napus* | A05-6NI | Canada | *B. napus* | A07-46NR | Canada |
| *B. napus* | A05-10NI | Canada | *B. napus* | A07-47NR | Canada |
| *B. napus* | A05-17NI | Canada | *B. napus* | A07-29NI | Canada |
| ***Panel 3 (Winter type)*** | | | *B. napus* | A07-33NI | Canada |
| *B. rapa* | Largo | Estonia | *B. napus* | A07-35NI | Canada |
| *B. rapa* | Prisma | Estonia | *B. napus* | 1CA1745.068 | Canada |
| *B. rapa* | JSv 01-13102 | Estonia | *B. napus* | 1CA1745.086 | Canada |
| *B. rapa* | JSv 01-11449 | Estonia | *B. napus* | 1CA1745.095 | Canada |
| *B. rapa* | JSv 01-11403 | Estonia | *B. napus* | 1RA1638.100 | Canada |
| *B. rapa* | JSv 00-15588 | Estonia | *B. napus* | 1RA1638.101 | Canada |
| *B. rapa* | JSv 00-13426 | Estonia | *B. napus* | 1RA1638.102 | Canada |
| *B. rapa* | Tianyou-4 | China | *B. napus* | 1RA1638.103 | Canada |
| *B. rapa* | Tianyou-7 | China | *B. napus* | 1RA1951.067 | Canada |
| *B. rapa* | 1-200119 | China | *B. napus* | 1RA1951.070 | Canada |
| | | | *B. napus* | 1RA1951.072 | Canada |
| | | | *B. napus* | 1RA1951.073 | Canada |
| | | | *B. napus* | 1RA1951.078 | Canada |
| | | | *B. napus* | 1RA1951.081 | Canada |

[1]
Estonia = Jogeva Plant Breeding Institute, Estonia; China = Gansu Agricultural University, China; Canada = University of Alberta, Canada, Europe = Different European countries, such as Germany, France, Denmark and United Kingdom.

[2]Spring growth habit recombinant inbred *B. napus* lines derived from Winter × Spring *B. napus* crosses.

used as queries in BlastN searches against NCBI genome assembly of *B. napus* (v1.0). If a pre- miRNA sequence was mapped (> 95% sequence identity) with *B. napus* genome assembly, corresponding "hit" sequences along with 500 bp from both 5'and 3'flanking regions were extracted from the assembly. The primary miRNA (pri-miRNA) sequences of varying lengths, comprising of the pre-miRNA sequence and flanking sequences, were scanned for repeats using a standalone program, WebSat (http://wsmartins.net/websat/) (Martins *et al.* 2009). Subsequently, repeat motif lengths ≥10 for mono- and ≥7 for di-nucleotide (nt) repeat were employed for designing SSR primers from primary miRNA gene sequences using WebSat with default parameters.

### 4.2.3 Primer selection and PCR amplification

A total of 31 primer pairs, located on different chromosomes were selected for further validation (Table 4.2). A universal M13 sequence tag was attached to the 5'end of the forward primer in each set (Table 4.2). Amplification reactions were performed in a final volume of 12 µL containing 50 ng of genomic DNA, 1.25 µL of 10X Taq-buffer, 0.125 µL of 0.2 mM dNTPs, 1.25 µL of 50 mM $MgCl_2$, 0.25 µL of 0.2 nM fluorescent epitope (FAM/ VIC/ NED/ or PET) tagged M13 primer (Applied Biosystems) and 0.15 µL of 5U/µL of Platinum Taq Polymerase (Thermo Scientific). PCR reactions were performed using following conditions: an initial denaturation step at 95 °C for 3 min, followed by 35 or 40 cycles of 95 °C for 30 s, 53 or 55 °C for 30 s and 72 °C for 30 s. The final extension was at 72 °C for 7 min and PCR products were frozen at -20 °C, until further analysis. PCR products were resolved by capillary electrophoresis, using 3730 DNA analyzer (Applied Biosystems) and amplification product sizes were determined using GeneMapper

v 4.0 software. Six primer pairs were excluded from the study due to lack of PCR products and / or due to weak amplification.

### 4.2.4 Marker analysis

Scoring of SSR markers was based on the amplified fragment, with 1 or 0 assigned based on presence or absence of an amplicon of a given size. Polymorphism Information Content (PIC; Xu 2010) values were calculated using the following formula, where for q represents null allele frequency and p represents allele frequency.

$q = [\text{no. of individuals lacking amplicon} / \text{total no. of individuals}]^{1/2}$
$p = 1 - q$
$PIC = 1 - \sum p^2$

Jaccard similarity co-efficient matrix was calculated using Darwin v5.0 and dendogram displaying relationship among 64 accessions was constructed using Neighbor-Joining method (Perrier 2003; Saitou and Nei 1987). SSR primers, which did not amplify, were excluded and a final set of 25 SSR primers pairs that produced clear polymorphisms were used for cluster analysis (Table 4.3).

## 4.3 Results

### 4.3.1 Identification of *B. napus* miR-SSRs

Out of 90 precursor miRNA sequences downloaded from miRBase, 41 sequences were excluded due to the absence of any repeat sequences, the rest of the sequences generated mono-, di- and tri-nt repeats with di-nt repeated 18 times and mono-nt repeated 21 times. Out of 49 SSR containing miRNA genes, $(T)_n$ was found to be present in maximum

frequency (22.5%) of miRNA genes, followed by 20.4 % for $(TC)_n$ and 16.3 % for $(CT)_n$. Only two miR-SSR possessing miRNA genes had tri-nt repeats $(ATC)_n$ and $(CAG)_n$. In addition, out of the total 49 miR-SSRs, 31 (63.2 %) were found to be present in the A genome, while 18 (36.7 %) were present in the C genome. It was observed that chromosome A1 possessed the highest number (6) of miRNA repeat motifs while chromosome A6 and A10 had the lowest number (1) of repeat motifs. In the C genome, chromosome C3 and C4 showed highest (5) and lowest (1) number of repeat motifs (Figure 4.1) while chromosome C1, C2 and C9 lacked miR-SSRs (Figure 4.1).

### 4.3.2 Validation of miR-SSRs

Primers were designed from 31 miR-SSRs with ≥10 for mono- and ≥7 for di-nt repeats and were tested on four different panels (Table 4.1), spring *B. napus* cultivars/lines (panel 1), winter *B. napus* cultivars (panel 2), winter *B. rapa* cultivars/lines (panel 3) and *B. napus* RILs (panel 4), to investigate the utility of these markers. Most of the primers amplified more than one locus; therefore, only clear bands with sharp peaks in the sequencing chromatogram were considered, while ambiguous or weak bands were not included in the analysis. Out of these 31 miR-SSRs, six primer pairs did not amplify properly and hence were excluded from further analysis. A total of 100 alleles were scored with 25 SSRs where 90 were polymorphic accounting for an average of 3.6 polymorphic alleles/marker. While miR156b-SSR generated highest number of 11 alleles, followed by miR166a-SSR which generated 8 alleles; and miR167b-SSR, miR166e-SSR, miR396a-SSR, miR399b-SSR and miR6030-SSR each generated the least number (two) of alleles (Table 4.3). The

lowest amplicon size (105 bp) was produced by miR393-SSR, and the highest amplicon size (446 bp) was produced by miR396a-SSR.

Differences in molecular size between the smallest and largest allele for a given SSR varied from 6 bp (miR166e-SSR) to 298 bp (miR396a-SSR) reflecting a huge variation among repeat regions of different alleles (Table 4.3). Allelic variation of *B. napus* miR-SSR markers was evaluated by determining PIC values. The average PIC value for the 24 miR-SSRs was 0.72, with highest PIC value (0.99) for miR166e-SSR and the lowest 0.04) for miR156e-SSR (Table 4.3). PIC value of 88% of the markers was more than 0.50. In addition, the average PIC value (0.58) of the RILs was lower than the winter *B. napus* (0.74), spring *B. napus* (0.72) and winter *B. rapa* (0.690), indicating that miRNAs belonging to RILs were less diverse than other three panels. In the 64 *Brassica* lines used in this study, the miR159-SSR amplified four alleles of 396 bp, 405 bp, 411 bp and 423 bp with PIC value of 0.45 (Table 4.4). The 411 bp allele was amplified in all winter *B. napus* and *B. rapa* accessions, except the winter *B. rapa* accession JSv 01-11403; however, it was absent in all 20 spring *B. napus* cultivars/lines. The RILs were developed from winter × spring *B. napus* crosses, therefore, the 411 bp allele could be detected in some of the RILs while it was absent in other. Thus, out of 25 miR-SSRs used in this study, miR159-SSR was able to differentiate between spring and winter *Brassica* accessions.

### 4.3.3 Cluster analysis

The unweighted Neighbour-Joining based dendogram constructed using the binary miR-SSR data divided the lines into five clusters/groups (Figure 4.2). In general, the accessions with known cold tolerance or genetic diversity groups were grouped together in

the dendogram. The clusters I, II and IV comprised lines of winter *B. rapa*, winter *B. napus* and spring *B. napus* respectively; while the RILs, which were derived from crossing of the winter and spring type *B. napus*, formed intermediate and distinct clusters (Cluster III and V, Figure 4.2).

The RILs produced from the same cross often sub-clustered together (Figure 4.2); for example, A07-45NR, A07-46NR and A07-47NR were produced from the same cross between spring and winter type and formed as sub-cluster within cluster III. In contrast, some RILs produced from the same cross segregated into clusters III and V, such as 1RA1951.067and 1RA1951.070 formed a sub-cluster in cluster III, while 1RA1951.072, 1RA1951.073, 1RA1951.078 and 1RA1951.081 formed a separate sub-cluster in cluster V (Figure 4.2) due to segregation of the markers alleles. For the same reason, the line A07-38NR clustered with spring *B. napus* (Figure 4.2). The Jaccard's similarity index between pairs of *Brassica* accessions ranged from 21 % to 86 % with a mean similarity index of 54 % (Supplementary File 1). The mean similarity index of winter *B. rapa*, winter *B. napus,* spring *B. napus* and RILS was 57.2 %, 47.32 %, 52.9% and 56.5 %, respectively. Moreover, two RILs, 1RA1951.081 and 1RA1951.078 showed a high similarity index of 86 %.

**Table 4.2: List of primers used in this study.**

| miRNA-SSR | Forward Primer (5'-3')* | Reverse Primer (5'-3') |
|---|---|---|
| **miR399b-SSR** | CTTGTTGTGTGCTACGGATTCT | CAAGTAATGGTTTCCTGCCAAT |
| **miR6030-SSR** | GTGGAGAATGGAATGTGATGAA | CCATAGCTTAACCCGAGTGAGT |
| **miR167b-SSR** | TGAGGCCAGTTACACAAGAAAA | AAAATTAGGGTTTAAGGGCGAG |
| **miR396a-SSR** | GTTAATGTGGCAATGGAATGGT | GGATCTTCATGTTCTCCACCTC |
| **miR166e-SSR** | ATAATAGCAACCCGAGCTTTTG | ATTCTCCACTCCACTTGTCTTTC |
| **miR156a-SSR** | CTAGTGCTGATCTCTTTGGCCT | AGTAGGGAGCTGGGGATTAAAA |
| **miR399c-SSR** | GGCCACAAAATATCAGAAGCAT | TCCAAGAATGTAGTATCCACTTCG |
| **miR169m-SSR** | GTTGATTTCTTACGACGCCTTT | TGGCAAGCTCTTACTCTTGATG |
| **miR156f-SSR** | GCGACAAAAGCCATAAAGAAAG | AATTCAGACACCCTTTGGAAGA |
| **miR394a-SSR** | ACGTTGTGTTTTGTGTGAGGAG | ACCGCCATTGAGAATTTATGAG |
| **miR159-SSR** | GATGGTTTATGTATGCTGTGGC | TCCTCACATTCCAACACTGAAC |
| **miR171g-SSR** | CTCTTTGATATTGGCCTGGTTC | GGAAAGGAAGCTAATGAAGGGT |
| **miR164d-SSR** | ACGTAAACGAGCAAGCAGAAGT | CATAGTCGGAAGGGGAGATACA |
| **miR172c-SSR** | CTAGCCTCTGCTCCTCACATTT | CCACAGACGAAAGACCCTAATC |
| **miR393-SSR** | TTGTTGGAGATGCGTTCAAGT | TTCCACTTTGAGGGTTCCTTTA |
| **miR169g-SSR** | GTCTGTGGATCTTGTCGCCTAT | GAGCTTAATTGCCCTTGTGTTT |
| **miR172b-SSR** | GGGCTTGTTTTGTATTGATGTG | GAGGCTAGGTCTTTTGCCTTTT |
| **miR171a-SSR** | GCCAATATCACGCATATAACCA | ATAGCAAACCACGACAACATGA |
| **miR156g-SSR** | TACTTGCCTTAACCCACCGTAT | ACAGGGCCAGCTCAAGAAT |
| **miR167c-SSR** | GCACCCTTAAACCCTAATTTCC | CTCAGAAGCCCTAGCCAAACTA |
| **miR164b-SSR** | CTGGAAGCTGAGAAGAAGTGAA | CCCTATCTAGTCCACACCCAAC |
| **miR166f-SSR** | TCATTCCCTCATCATAACACCA | CATTCCCCTCAACTGAAATAGC |
| **miR156e-SSR** | AGGTGTGCTCTTCTACCCAGTC | GATGAGTATTGCTTTCTGCCAA |
| **miR166a-SSR** | GAGAGAGGGACAGAGAGTGTGG | GAGAGAGGGACAGAGAGTGTGG |
| **miR156b-SSR** | AGGTTTGAGAGTGATGCTGGTT | GGTGACAGAAGTATAGAGAGCACG |

*M13 sequence (5'-CACGACCGTTGTAAAACGAC-3') attached to 5'of each forward primer

**Table 4.3**: **Summary of miR-SSR markers tested in 64 *Brassica* accessions to study the utility of this new marker type for genotyping.**

| miRNA-SSR | Chromosome Number | Alleles | Repeat Motif | Amplicon Size (bp) | PIC |
|---|---|---|---|---|---|
| miR399b-SSR | A5 | 2 | $(A)_{10}$ | 324-338 (14) | 0.9602 |
| miR6030-SSR | A6 | 2 | $(T)_{10}$ | 234-247 (13) | 0.5514 |
| miR167b-SSR | A5 | 2 | $(CT)_{12}$ | 243-251 (8) | 0.9867 |
| miR396a-SSR | A1 | 2 | $(CT)_{18}$ | 148-446 (298) | 0.7827 |
| miR166e-SSR | A4 | 2 | $(A)_{11}$ | 204-210 (6) | 0.999 |
| miR156a-SSR | A4 | 3 | $(TC)_{9}$ | 386-393 (7) | 0.4759 |
| miR399c-SSR | C6 | 3 | $(T)_{13}$ | 400-413 (13) | 0.5538 |
| miR169m-SSR | A8 | 3 | $(T)_{11}$ | 345-364 (19) | 0.7924 |
| miR156f-SSR | A9 | 3 | $(A)_{11}$ | 197-206 (9) | 0.8998 |
| miR394a-SSR | A8 | 3 | $(T)_{11}$ | 387-414 (27) | 0.5686 |
| miR159-SSR | C6 | 4 | $(A)_{11}$ | 396-423 (27) | 0.4545 |
| miR171g-SSR | A3 | 4 | $(CT)_{8}$ | 200-414 (214) | 0.7947 |
| miR164d-SSR | C7 | 4 | $(CT)_{18}$ | 112-402 (290) | 0.7853 |
| miR172c-SSR | A2 | 4 | $(CT)_{8}$ | 312-324 (14) | 0.6407 |
| miR393-SSR | A7 | 4 | $(T)_{10}$ | 105-272 (167) | 0.7665 |
| miR169g-SSR | C5 | 4 | $(T)_{21}$ | 152-299 (147) | 0.6416 |
| miR172b-SSR | C3 | 4 | $(TC)_{16}$ | 177-456 (279) | 0.71 |
| miR171a-SSR | C8 | 4 | $(A)_{13}$ | 193-402 (209) | 0.8713 |
| miR156g-SSR | C6 | 4 | $(TA)_{13}$ | 162-325 (163) | 0.649 |
| miR167c-SSR | A3 | 5 | $(T)_{11}$ | 365-386 (21) | 0.6637 |
| miR164b-SSR | A4 | 5 | $(T)_{11}$ | 129-360 (231) | 0.6926 |
| miR166f-SSR | C5 | 5 | $(TC)_{7}$ | 159-174 (15) | 0.9561 |
| miR156e-SSR | C3 | 5 | $(TC)_{9}$ | 189-250 (61) | 0.0045 |
| miR166a-SSR | A5 | 8 | $(TC)_{7}$ | 102-372 (270) | 0.8983 |
| miR156b-SSR | A3 | 11 | $(AG)_{8}$ | 160-419 (259) | 0.9652 |

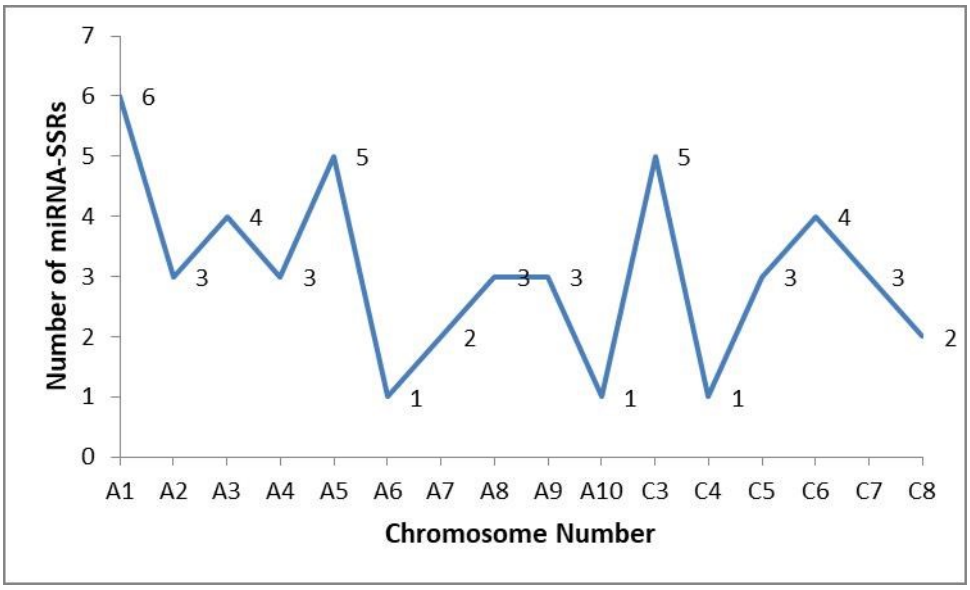



**Figure 4.1: Distribution of miRNA-Simple Sequence Repeats (SSR) on different chromosomes of *B. napus.***

**4.4 Discussion**

SSRs had been reported to play important biological functions in the regulation of chromatin organization, DNA metabolic processes, gene activity and RNA structure (Li *et al.* 2004; Vieira *et al.* 2016). SSRs have been increasingly identified and characterized to be intergenic and also present in untranslated (UTRs) regions of genes (Hancock and Simon 2005; Kashi and King 2006; Vieira *et al.* 2016). SSR variations in 5'-UTRs has been observed to regulate gene expression by affecting transcription and translation, while SSR expansions in the 3'-UTRs cause transcription slippage and produce expanded mRNA, which can disrupt splicing and affect other cellular functions (Vieira *et al.* 2016).

Since the discovery of miRNA in plants (Reinhart *et al.* 2002; Park *et al.* 2005), several studies have demonstrated their role in regulating the expression of genes/transcription factors during various abiotic stresses, including LT stress (Sun *et al*.

2015; Karimi *et al.* 2016, Megha *et al.* 2017). For instance, miR395 in plants species is known to regulate the expression of ATP sulfurylases (APS) that catalyze the primary step of intracellular sulfate activation (Jones-Rhoades and Bartel, 2004). The expression of APS gene was reported to be up-regulated in response to LT in *Glycine max* accompanied by an increase in antioxidant-glutathione (Phartiyal *et al.* 2006). Similarity, in different plant species exposed to LT stress, differential expression of various transcripts belonging to class of heat shock proteins, laccases, F-box protein and of TFs such as *NAC* (for *NAM/ATAF1, 2/CUC2),* Scarecrow-like, and HD-Zip have been reported and these targets are also known to be targets of miRNAs (Chen *et al*. 2012, Karimi *et al.* 2016, Xu *et al*. 2016).

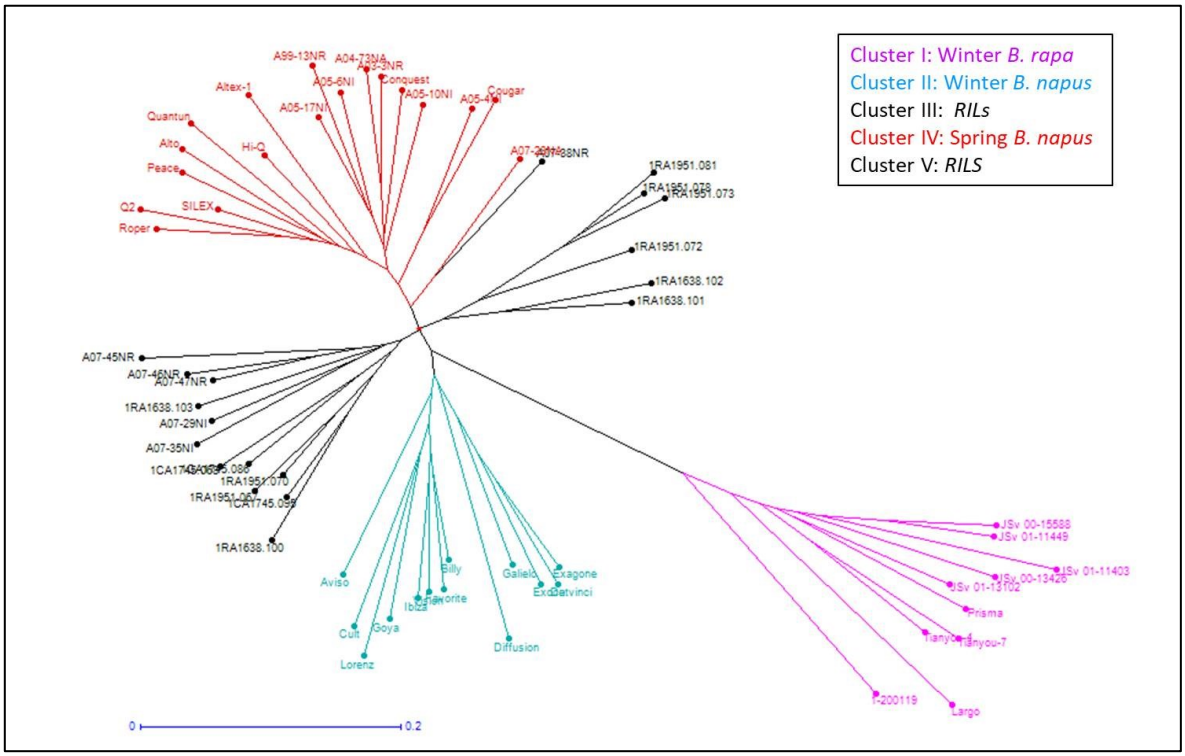



**Figure 4.2**: **Phylogram depicting genetic similarity of 64 accessions of Brassica based on 90 polymorphic alleles amplified by 25 miRNA-SSR markers**

**Table 4.4**: **Distribution of different alleles amplified by miR159-SSR in *Brassica* accessions.**

| | Spring *B. napus* (20) | Winter *B. napus* (14) | Winter *B. rapa* (12) | RILs* (20) |
|---|---|---|---|---|
| 396 bp | 0 | 2 | 10 | 3 |
| 405 bp | 19 | 13 | 0 | 20 |
| 411 bp | 0 | 14 | 9 | 13 |
| 423 bp | 20 | 0 | 0 | 4 |

*Recombinant inbred lines derived from Winter × Spring *B. napus* crosses.

Thus, a role for miRNAs in mediating plant responses to abiotic stresses including CS is becoming increasingly clear.

Because of the aforementioned roles for miRNAs in mediating CS responses in plants, we initiated a study to determine whether polymorphisms present in the miRNA genes like SSRs can be used to determine genetic variability between *Brassica* lines belonging to winter and spring growth habit types. Although this is the first report of SSRs from the non-coding miRNA genes of *Brassica*, there have been reports of SSRs in rice miRNA genes (Mondal and Gaine, 2014; Ganie and Mondal, 2015). In rice, 12 miR-SSR markers showed clear polymorphisms among the contrasting panels of salt tolerant and susceptible cultivars (Mondal and Gaine 2014). In addition, presence of (CT) dinucleotide SSR in one primary miRNA candidate overlapping the neighbouring *NAP1* gene of black pepper has been functionally validated (Joy and Soniya, 2012). However, as mentioned earlier, this is the first report where SSRs from the non-coding regions of miRNA genes have been used to study the potential of this type of marker for use in breeding and research.

Several studies have successfully developed and employed the miRNA-based marker system for genotyping purposes in different plants including, *Brassica* species (Fu *et al.* 2013), foxtail millet and related grass species (Yadav et al. 2014) and flax (Razna *et al.* 2015). Yadav *et al*. (2014), designed 66 markers from pre-miRNAs of foxtail millet; 100% of these markers showed amplification products in five cultivars, and the markers also showed a high level (≈ 67 %) of transferability among the millets and non-millets species. Although all of the above studies have developed miRNA-based markers for genotyping purposes, as of yet, there are no reports describing the development of trait specific miRNA-marker in plants.

Fu *et al.* (2013) designed 46 single miRNA-based markers from conserved sequences of *Brassica* pre-miRNAs and subsequently generated 34 primer pairs using random combination of these primers. These primer pairs were used to differentiate the six *Brassica* species of U's triangle, such as *B. napus*, *B. oleracea*, *B. rapa*, *B, juncea*, *B. carinata* and *B. nigra* (Fu *et al.* 2013). In breeding applications, it is also important to understand the extent of genetic diversity within a species or gene pool; therefore, we investigated the potential of miRNA markers through evaluating 64 accessions of *B. napus* and *B. rapa*. Following this approach, we were able to generate reliable estimates of genetic divergence among these accessions. The winter (cluster II) and spring *B. napus* (cluster IV) types are known to be genetically quite distinct (e.g. Hasan *et al*. 2006; Bus *et al.* 2011) and the genome of *B. rapa* (cluster I) is also known to be genetically distinct from the A genome of *B. napus* (e.g. Thormann *et al.* 1994; Xiao *et al*. 2010). The miR-SSR markers used in this study clearly differentiated these three gene pools demonstrating their value for use in breeding and research.

Of the miR-SSR markers used in this study, the marker based on miR159 (miR159-SSR) could be detected in 96% (23/24) of winter type accessions but were absent in 100% (20/20) of the spring *B. napus* accessions used in this study. The expression of mature miR159, which was present in all winter *B. napus* and winter *B. rapa* accessions (except winter *B. rapa* line JSv 01-11403), has been previously reported to be differentially regulated under CS in *A. thaliana*, wheat and *Medicago* (Zhou *et al.* 2008; Shu *et al.* 2016; Song *et al.* 2017). Winter and spring growth habit *Brassica* are not only different in vernalization requirement genes, but also with respect to the fact that winter types also harbor cold or freezing tolerance genes some of which can be inherited independently of the vernalization genes (Kole *et al*. 2002). A BLASTn search showed miR159-SSR to be present at 3407 bp to Alpha-Dioxygenase 2-like gene at 5'end and 6551 bp to Ethylene Insensitive3-like gene at the 3'end of chromosome C6 of *B. napus*. However, Kole *et al*. (2002) reported either winter survival or freezing tolerance QTL from most of the A genome chromosomes, as well as C7 and C9 chromosomes of the C genome. Among these, some of the chromosomes, such as A2 and A8, found to carry QTL for freezing tolerance and winter survival in the same genomic region, while no QTL for freezing tolerance was found in the other QTL regions associated with winter survival. Therefore, at this time, the association of miR159-SSR with cold tolerance cannot be attributed to a QTL; additional studies with this marker and additional markers from the same genomic region will be needed to further evaluate the relationship of this miRNA marker with cold tolerance in *Brassica*.

In the present investigation, all miRNA genes of *B. napus* present in miRBase were scanned and a total of 49 mono-, di-and tri- nt SSRs were mined. Previously, mono-nt

motif of A and C, di-nt motif of AT, AG, AC and CG have been found to be abundant in the assembled genomic sequences of *B. napus* (Cheng *et al*. 2009; Shi *et al*. 2014). However, in our study, mono-nt A and T, di-nt motif TC, CT and TA were most abundant. It has been previously reported that plant genomes have higher abundance of mono-, di- and tetra-nt microsatellites in the non-coding regions, whereas the tri- and hex-nt repeats were abundant in the coding regions across different plant species (Morgante *et al*. 2002). Copy number mutations in tri-nucleotide motifs cannot lead to frame shift mutation (Morgante *et al*. 2002) and the high abundance of mono- and di-nt repeat motifs in the miRNA genes might be indicative of the fact that these regions are more variable and prone to mutation.

Generally, PIC values of more than 0.5 are indicative of high levels of polymorphism among different genotypes, PIC values between 0.25 and 0.5 indicate medium polymorphism and values below 0.25 indicate low levels of polymorphism (Botstein *et al*. 1980). In the present study, the PIC values of miR-SSRs ranged from 0.04 to 0.99, with 88 % of markers exceeding 0.5 (Table 4.3). Previously, PIC values for miR-SSRs developed and used in rice ranged from 0 to 0.46 (Mondal and Gaine, 2014; Ganie and Mondal, 2015), while that of miRNA-based markers in *Brassica* ranged from 0.14 to 0.69 (Fu *et al*. 2013). Moreover, previous studies have established a varying range of PIC values for SSR markers in *Brassica* species, e.g., 0.14 to 0.69 (An et al. 2011), 0.58 to 0.99 (Hobson and Rahman, 2016) and 0.04 to 0.81 (Thakur *et al*. 2017). Thus, the miR-SSRs used in current study are highly polymorphic when compared to other reports utilizing miRNAs as markers or SSRs.

## Conclusion

In summary, miR-SSR molecular markers were designed from pre-miRNA flanking sequences and used to analyze genetic diversity of spring and winter growth habit *Brassica* accessions. These miR-SSR markers exhibited high polymorphism, and grouping of the *Brassica* accessions by cluster analysis was generally consistent with known pedigree suggesting the usefulness of this type of markers for use in breeding and research. We verified that there was repeat length variation in winter and spring *Brassica* as detected by miR-SSRs, and demonstrated that a SSR present within MIR159 was able to distinguish winter and spring growth habit *Brassica* accessions indicating its potential association with cold tolerance. This polymorphism can be linked to either differential regulation and processing of pre-miRNA from miRNA gene and thus culminating in a differential expression of mature miRNA. Development of highly polymorphic miRNA-based molecular markers, associated with specific traits, will offer advantages over other marker systems, to plant breeders for crop improvement programs.

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Supplementary files

**Supplementary file 1**: Jaccard dissimilarity index calculated by Darwin for 64 *Brassica* accessions

| A07-38NR | A07-38NR | A07-45NR | A07-46NR | A07-47NR | A07-29NI | A07-35NI | 1CA1745.0 | 1CA1745.0 | 1CA1745.0 | 1RA1638.1 | 1RA1638.101 | 1RA1638.1 | 1RA1638.1 | 1RA1951.0 | 1RA1951.0 | 1RA1951.0 | 1RA1951.0 | 1RA1951.0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A07-45NR | 0.33 | | | | | | | | | | | | | | | | | |
| A07-46NR | 0.29 | 0.31 | | | | | | | | | | | | | | | | |
| A07-47NR | 0.28 | 0.27 | 0.19 | | | | | | | | | | | | | | | |
| A07-29NI | 0.28 | 0.38 | 0.30 | 0.29 | | | | | | | | | | | | | | |
| A07-35NI | 0.30 | 0.36 | 0.25 | 0.24 | 0.26 | | | | | | | | | | | | | |
| 1CA1745.068-A2( | 0.31 | 0.43 | 0.35 | 0.27 | 0.34 | 0.29 | | | | | | | | | | | | |
| 1CA1745.086-A2( | 0.32 | 0.45 | 0.30 | 0.29 | 0.31 | 0.31 | 0.24 | | | | | | | | | | | |
| 1CA1745.095-A2( | 0.35 | 0.43 | 0.32 | 0.38 | 0.26 | 0.36 | 0.31 | 0.21 | | | | | | | | | | |
| 1RA1638.100-A2( | 0.34 | 0.40 | 0.34 | 0.37 | 0.37 | 0.39 | 0.35 | 0.28 | 0.20 | | | | | | | | | |
| 1RA1638.101-A2( | 0.29 | 0.37 | 0.29 | 0.32 | 0.38 | 0.41 | 0.35 | 0.30 | 0.27 | 0.24 | | | | | | | | |
| 1RA1638.102-A2( | 0.32 | 0.35 | 0.29 | 0.28 | 0.32 | 0.35 | 0.28 | 0.32 | 0.30 | 0.31 | 0.21 | | | | | | | |
| 1RA1638.103-A2( | 0.28 | 0.36 | 0.32 | 0.29 | 0.31 | 0.28 | 0.36 | 0.38 | 0.36 | 0.32 | 0.32 | 0.25 | | | | | | |
| 1RA1951.067-A2( | 0.34 | 0.35 | 0.24 | 0.28 | 0.30 | 0.30 | 0.33 | 0.30 | 0.22 | 0.26 | 0.31 | 0.24 | 0.28 | | | | | |
| 1RA1951.070-A2( | 0.29 | 0.35 | 0.29 | 0.28 | 0.25 | 0.27 | 0.30 | 0.30 | 0.22 | 0.29 | 0.31 | 0.24 | 0.27 | 0.15 | | | | |
| 1RA1951.072-A2( | 0.36 | 0.42 | 0.36 | 0.25 | 0.37 | 0.39 | 0.35 | 0.32 | 0.32 | 0.36 | 0.35 | 0.31 | 0.39 | 0.28 | 0.20 | | | |
| 1RA1951.073-A2( | 0.35 | 0.49 | 0.41 | 0.38 | 0.45 | 0.49 | 0.41 | 0.45 | 0.42 | 0.46 | 0.30 | 0.37 | 0.47 | 0.44 | 0.37 | 0.27 | | |
| 1RA1951.078-A2( | 0.41 | 0.47 | 0.45 | 0.35 | 0.44 | 0.44 | 0.33 | 0.44 | 0.44 | 0.47 | 0.33 | 0.34 | 0.42 | 0.39 | 0.36 | 0.25 | 0.15 | |
| 1RA1951.081-A2( | 0.38 | 0.47 | 0.45 | 0.40 | 0.46 | 0.46 | 0.38 | 0.46 | 0.46 | 0.47 | 0.33 | 0.34 | 0.42 | 0.43 | 0.40 | 0.33 | 0.18 | 0.14 |
| Altex-1 | 0.35 | 0.44 | 0.40 | 0.41 | 0.36 | 0.39 | 0.41 | 0.34 | 0.36 | 0.35 | 0.37 | 0.40 | 0.41 | 0.40 | 0.37 | 0.37 | 0.39 | 0.35 |
| Alto | 0.35 | 0.39 | 0.39 | 0.41 | 0.43 | 0.45 | 0.45 | 0.47 | 0.47 | 0.48 | 0.39 | 0.44 | 0.43 | 0.48 | 0.43 | 0.44 | 0.39 | 0.38 |
| Peace | 0.42 | 0.47 | 0.44 | 0.41 | 0.43 | 0.39 | 0.41 | 0.38 | 0.41 | 0.48 | 0.41 | 0.46 | 0.43 | 0.42 | 0.41 | 0.44 | 0.47 | 0.44 |
| Quantun | 0.42 | 0.44 | 0.42 | 0.43 | 0.37 | 0.46 | 0.46 | 0.47 | 0.43 | 0.51 | 0.44 | 0.47 | 0.41 | 0.42 | 0.42 | 0.47 | 0.46 | 0.43 |
| Q2 | 0.34 | 0.38 | 0.41 | 0.46 | 0.37 | 0.42 | 0.49 | 0.46 | 0.39 | 0.43 | 0.42 | 0.43 | 0.42 | 0.43 | 0.40 | 0.47 | 0.46 | 0.48 |
| Hi-Q | 0.34 | 0.42 | 0.36 | 0.37 | 0.32 | 0.35 | 0.35 | 0.30 | 0.30 | 0.38 | 0.31 | 0.38 | 0.35 | 0.38 | 0.33 | 0.40 | 0.43 | 0.38 |
| SILEX | 0.38 | 0.42 | 0.38 | 0.42 | 0.35 | 0.40 | 0.42 | 0.37 | 0.31 | 0.41 | 0.38 | 0.43 | 0.42 | 0.39 | 0.34 | 0.38 | 0.42 | 0.39 |
| Roper | 0.39 | 0.42 | 0.41 | 0.46 | 0.40 | 0.42 | 0.45 | 0.44 | 0.38 | 0.45 | 0.42 | 0.47 | 0.44 | 0.45 | 0.41 | 0.47 | 0.44 | 0.44 |
| A07-28NA | 0.23 | 0.36 | 0.32 | 0.32 | 0.31 | 0.36 | 0.36 | 0.36 | 0.31 | 0.35 | 0.32 | 0.35 | 0.33 | 0.35 | 0.27 | 0.34 | 0.33 | 0.37 |
| A99-13NR(lobele | 0.29 | 0.38 | 0.38 | 0.42 | 0.35 | 0.42 | 0.46 | 0.43 | 0.37 | 0.36 | 0.38 | 0.41 | 0.35 | 0.38 | 0.33 | 0.40 | 0.42 | 0.45 |
| A03-3NR | 0.31 | 0.43 | 0.37 | 0.36 | 0.38 | 0.38 | 0.43 | 0.40 | 0.40 | 0.46 | 0.39 | 0.44 | 0.36 | 0.37 | 0.35 | 0.35 | 0.41 | 0.40 |
| Cougar | 0.31 | 0.46 | 0.38 | 0.37 | 0.32 | 0.39 | 0.40 | 0.29 | 0.27 | 0.28 | 0.30 | 0.43 | 0.37 | 0.33 | 0.30 | 0.33 | 0.39 | 0.43 |
| A05-4NI | 0.23 | 0.41 | 0.33 | 0.32 | 0.31 | 0.31 | 0.34 | 0.29 | 0.31 | 0.35 | 0.32 | 0.35 | 0.36 | 0.30 | 0.25 | 0.27 | 0.36 | 0.38 |
| A05-6NI | 0.29 | 0.39 | 0.36 | 0.41 | 0.36 | 0.39 | 0.43 | 0.36 | 0.34 | 0.42 | 0.31 | 0.38 | 0.39 | 0.36 | 0.31 | 0.38 | 0.37 | 0.40 |
| A05-10NI | 0.32 | 0.44 | 0.42 | 0.39 | 0.35 | 0.41 | 0.42 | 0.35 | 0.35 | 0.38 | 0.33 | 0.34 | 0.30 | 0.36 | 0.33 | 0.38 | 0.41 | 0.34 |
| A05-17NI | 0.32 | 0.40 | 0.36 | 0.40 | 0.35 | 0.35 | 0.44 | 0.37 | 0.33 | 0.38 | 0.36 | 0.38 | 0.35 | 0.34 | 0.32 | 0.38 | 0.42 | 0.39 |
| Conquest | 0.35 | 0.45 | 0.43 | 0.41 | 0.40 | 0.38 | 0.41 | 0.38 | 0.34 | 0.44 | 0.41 | 0.42 | 0.38 | 0.39 | 0.35 | 0.37 | 0.41 | 0.38 |
| A04-73NA | 0.36 | 0.45 | 0.40 | 0.39 | 0.34 | 0.39 | 0.43 | 0.41 | 0.36 | 0.44 | 0.45 | 0.44 | 0.39 | 0.38 | 0.37 | 0.38 | 0.47 | 0.45 |
| Da vinci | 0.35 | 0.43 | 0.39 | 0.34 | 0.38 | 0.43 | 0.39 | 0.31 | 0.38 | 0.42 | 0.36 | 0.39 | 0.45 | 0.44 | 0.39 | 0.39 | 0.36 | 0.37 |
| Galielo | 0.33 | 0.39 | 0.38 | 0.32 | 0.36 | 0.41 | 0.39 | 0.32 | 0.39 | 0.38 | 0.35 | 0.40 | 0.34 | 0.44 | 0.42 | 0.38 | 0.37 | 0.36 |
| Exocet | 0.32 | 0.43 | 0.41 | 0.36 | 0.42 | 0.45 | 0.38 | 0.36 | 0.42 | 0.39 | 0.38 | 0.45 | 0.36 | 0.41 | 0.41 | 0.43 | 0.40 | 0.39 |
| Exagone | 0.26 | 0.44 | 0.36 | 0.32 | 0.39 | 0.44 | 0.37 | 0.32 | 0.39 | 0.38 | 0.35 | 0.42 | 0.41 | 0.42 | 0.40 | 0.38 | 0.34 | 0.41 |
| Ibiza | 0.39 | 0.47 | 0.39 | 0.34 | 0.40 | 0.40 | 0.29 | 0.31 | 0.38 | 0.41 | 0.34 | 0.41 | 0.36 | 0.41 | 0.34 | 0.41 | 0.40 | 0.42 |
| Lorenz | 0.46 | 0.48 | 0.44 | 0.39 | 0.41 | 0.46 | 0.39 | 0.36 | 0.39 | 0.47 | 0.43 | 0.44 | 0.43 | 0.44 | 0.42 | 0.44 | 0.46 | 0.47 |
| Favorite | 0.43 | 0.44 | 0.36 | 0.29 | 0.42 | 0.42 | 0.31 | 0.31 | 0.35 | 0.34 | 0.34 | 0.43 | 0.39 | 0.39 | 0.36 | 0.36 | 0.40 | 0.39 |
| Billy | 0.39 | 0.44 | 0.37 | 0.29 | 0.38 | 0.38 | 0.32 | 0.31 | 0.33 | 0.39 | 0.32 | 0.37 | 0.31 | 0.39 | 0.34 | 0.39 | 0.36 | 0.35 |
| Vision | 0.37 | 0.45 | 0.39 | 0.29 | 0.36 | 0.36 | 0.29 | 0.33 | 0.40 | 0.41 | 0.38 | 0.39 | 0.36 | 0.35 | 0.30 | 0.37 | 0.38 | 0.37 |
| Verona | 0.46 | 0.49 | 0.48 | 0.39 | 0.45 | 0.47 | 0.39 | 0.41 | 0.43 | 0.44 | 0.41 | 0.44 | 0.38 | 0.46 | 0.43 | 0.42 | 0.41 | 0.35 |
| Aviso | 0.44 | 0.46 | 0.41 | 0.38 | 0.42 | 0.46 | 0.38 | 0.33 | 0.33 | 0.39 | 0.32 | 0.34 | 0.39 | 0.41 | 0.38 | 0.38 | 0.40 | 0.34 |
| Diffusion | 0.42 | 0.42 | 0.42 | 0.43 | 0.47 | 0.52 | 0.50 | 0.43 | 0.41 | 0.42 | 0.39 | 0.42 | 0.47 | 0.45 | 0.44 | 0.42 | 0.41 | 0.45 |
| Cult | 0.47 | 0.47 | 0.49 | 0.42 | 0.40 | 0.45 | 0.43 | 0.33 | 0.33 | 0.44 | 0.45 | 0.44 | 0.40 | 0.44 | 0.41 | 0.36 | 0.49 | 0.44 |
| Largo | 0.76 | 0.68 | 0.72 | 0.67 | 0.65 | 0.67 | 0.70 | 0.67 | 0.69 | 0.70 | 0.73 | 0.72 | 0.71 | 0.69 | 0.71 | 0.69 | 0.74 | 0.70 |
| Prisma | 0.69 | 0.63 | 0.66 | 0.65 | 0.64 | 0.67 | 0.67 | 0.68 | 0.62 | 0.67 | 0.67 | 0.69 | 0.66 | 0.65 | 0.64 | 0.64 | 0.69 | 0.68 |
| JSv 01-13102 | 0.68 | 0.62 | 0.63 | 0.61 | 0.61 | 0.63 | 0.62 | 0.61 | 0.63 | 0.64 | 0.66 | 0.64 | 0.63 | 0.64 | 0.65 | 0.63 | 0.71 | 0.68 |
| JSv 01-11449 | 0.71 | 0.63 | 0.66 | 0.65 | 0.68 | 0.65 | 0.67 | 0.66 | 0.64 | 0.67 | 0.70 | 0.71 | 0.68 | 0.65 | 0.66 | 0.64 | 0.71 | 0.69 |
| JSv 01-11403 | 0.76 | 0.70 | 0.71 | 0.68 | 0.67 | 0.67 | 0.72 | 0.71 | 0.69 | 0.74 | 0.73 | 0.72 | 0.69 | 0.70 | 0.69 | 0.71 | 0.76 | 0.74 |
| JSv 00-15588 | 0.69 | 0.60 | 0.65 | 0.63 | 0.67 | 0.63 | 0.62 | 0.67 | 0.65 | 0.70 | 0.70 | 0.67 | 0.67 | 0.67 | 0.66 | 0.65 | 0.69 | 0.66 |
| JSv 00-13426 | 0.68 | 0.61 | 0.68 | 0.67 | 0.64 | 0.67 | 0.70 | 0.68 | 0.66 | 0.67 | 0.68 | 0.71 | 0.66 | 0.65 | 0.66 | 0.68 | 0.73 | 0.70 |
| Tianyou-4 | 0.65 | 0.60 | 0.65 | 0.63 | 0.59 | 0.63 | 0.64 | 0.63 | 0.59 | 0.65 | 0.64 | 0.65 | 0.65 | 0.59 | 0.58 | 0.59 | 0.69 | 0.68 |
| Tianyou-7 | 0.69 | 0.66 | 0.69 | 0.65 | 0.67 | 0.67 | 0.66 | 0.69 | 0.65 | 0.67 | 0.68 | 0.69 | 0.69 | 0.65 | 0.66 | 0.63 | 0.69 | 0.66 |
| 1-200119 | 0.67 | 0.69 | 0.72 | 0.68 | 0.70 | 0.68 | 0.66 | 0.68 | 0.67 | 0.68 | 0.64 | 0.66 | 0.67 | 0.64 | 0.67 | 0.65 | 0.62 | 0.59 |

**Supplementary file 1** (cont.): Jaccard dissimilarity index calculated by Darwin for 64 *Brassica* accessions

| 1RA1951.( | Altex | Alto | Peace | Quantun | Q2 | Hi-Q | SILEX | Roper | A07-28NA | A99-13NR | A03-3NR | A06-9NR | A05-4NI | A05-6NI | A05-10NI | A05-17NI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.33 | | | | | | | | | | | | | | | | |
| 0.40 | | | | | | | | | | | | | | | | |
| 0.30 | 0.34 | | | | | | | | | | | | | | | |
| 0.40 | 0.37 | 0.24 | | | | | | | | | | | | | | |
| 0.45 | 0.35 | 0.30 | 0.30 | | | | | | | | | | | | | |
| 0.46 | 0.27 | 0.28 | 0.33 | 0.28 | | | | | | | | | | | | |
| 0.41 | 0.28 | 0.31 | 0.26 | 0.24 | 0.29 | | | | | | | | | | | |
| 0.37 | 0.28 | 0.24 | 0.21 | 0.26 | 0.22 | 0.17 | | | | | | | | | | |
| 0.41 | 0.25 | 0.23 | 0.26 | 0.26 | 0.21 | 0.25 | 0.20 | | | | | | | | | |
| 0.39 | 0.36 | 0.38 | 0.40 | 0.36 | 0.35 | 0.26 | 0.31 | 0.33 | | | | | | | | |
| 0.43 | 0.35 | 0.35 | 0.40 | 0.38 | 0.31 | 0.38 | 0.32 | 0.37 | 0.30 | | | | | | | |
| 0.35 | 0.49 | 0.32 | 0.32 | 0.37 | 0.42 | 0.35 | 0.29 | 0.38 | 0.34 | 0.26 | | | | | | |
| 0.43 | 0.30 | 0.42 | 0.37 | 0.38 | 0.33 | 0.31 | 0.31 | 0.36 | 0.37 | 0.31 | 0.35 | | | | | |
| 0.35 | 0.32 | 0.34 | 0.37 | 0.35 | 0.35 | 0.31 | 0.31 | 0.33 | 0.29 | 0.33 | 0.27 | 0.21 | | | | |
| 0.36 | 0.35 | 0.30 | 0.30 | 0.30 | 0.28 | 0.26 | 0.19 | 0.26 | 0.25 | 0.24 | 0.20 | 0.28 | 0.20 | | | |
| 0.31 | 0.30 | 0.28 | 0.31 | 0.29 | 0.31 | 0.25 | 0.25 | 0.30 | 0.32 | 0.29 | 0.26 | 0.31 | 0.28 | 0.24 | | |
| 0.39 | 0.33 | 0.31 | 0.26 | 0.29 | 0.30 | 0.21 | 0.16 | 0.30 | 0.29 | 0.20 | 0.22 | 0.34 | 0.29 | 0.17 | 0.21 | |
| 0.40 | 0.39 | 0.34 | 0.27 | 0.32 | 0.33 | 0.26 | 0.24 | 0.29 | 0.34 | 0.35 | 0.25 | 0.35 | 0.34 | 0.30 | 0.21 | 0.19 |
| 0.42 | 0.46 | 0.30 | 0.28 | 0.33 | 0.33 | 0.33 | 0.27 | 0.34 | 0.36 | 0.29 | 0.18 | 0.35 | 0.32 | 0.31 | 0.29 | 0.20 |
| 0.44 | 0.41 | 0.47 | 0.49 | 0.46 | 0.53 | 0.43 | 0.48 | 0.47 | 0.40 | 0.44 | 0.38 | 0.42 | 0.41 | 0.43 | 0.37 | 0.44 |
| 0.33 | 0.35 | 0.37 | 0.41 | 0.42 | 0.45 | 0.33 | 0.38 | 0.38 | 0.34 | 0.42 | 0.37 | 0.40 | 0.39 | 0.39 | 0.31 | 0.40 |
| 0.44 | 0.43 | 0.45 | 0.47 | 0.43 | 0.50 | 0.41 | 0.46 | 0.46 | 0.38 | 0.43 | 0.40 | 0.41 | 0.43 | 0.43 | 0.37 | 0.41 |
| 0.43 | 0.40 | 0.42 | 0.46 | 0.47 | 0.47 | 0.44 | 0.45 | 0.47 | 0.39 | 0.38 | 0.37 | 0.33 | 0.39 | 0.40 | 0.40 | 0.44 |
| 0.44 | 0.45 | 0.47 | 0.43 | 0.43 | 0.52 | 0.35 | 0.39 | 0.46 | 0.40 | 0.45 | 0.40 | 0.37 | 0.41 | 0.41 | 0.39 | 0.41 |
| 0.47 | 0.57 | 0.52 | 0.48 | 0.49 | 0.59 | 0.46 | 0.47 | 0.57 | 0.43 | 0.51 | 0.43 | 0.49 | 0.48 | 0.48 | 0.44 | 0.46 |
| 0.45 | 0.49 | 0.48 | 0.42 | 0.45 | 0.51 | 0.36 | 0.41 | 0.49 | 0.35 | 0.45 | 0.42 | 0.36 | 0.42 | 0.44 | 0.43 | 0.41 |
| 0.39 | 0.41 | 0.46 | 0.38 | 0.41 | 0.45 | 0.30 | 0.35 | 0.42 | 0.29 | 0.39 | 0.38 | 0.37 | 0.42 | 0.38 | 0.32 | 0.33 |
| 0.44 | 0.43 | 0.49 | 0.43 | 0.45 | 0.48 | 0.39 | 0.44 | 0.46 | 0.33 | 0.41 | 0.40 | 0.39 | 0.41 | 0.43 | 0.39 | 0.41 |
| 0.42 | 0.46 | 0.47 | 0.41 | 0.39 | 0.53 | 0.35 | 0.42 | 0.47 | 0.40 | 0.48 | 0.43 | 0.44 | 0.47 | 0.49 | 0.35 | 0.38 |
| 0.37 | 0.42 | 0.38 | 0.33 | 0.38 | 0.47 | 0.32 | 0.37 | 0.43 | 0.39 | 0.49 | 0.44 | 0.45 | 0.44 | 0.44 | 0.30 | 0.39 |
| 0.45 | 0.40 | 0.46 | 0.46 | 0.47 | 0.49 | 0.42 | 0.43 | 0.47 | 0.41 | 0.47 | 0.49 | 0.47 | 0.48 | 0.44 | 0.44 | 0.44 |
| 0.49 | 0.43 | 0.51 | 0.43 | 0.46 | 0.53 | 0.39 | 0.42 | 0.46 | 0.44 | 0.52 | 0.47 | 0.44 | 0.45 | 0.47 | 0.39 | 0.43 |
| 0.74 | 0.72 | 0.75 | 0.70 | 0.73 | 0.74 | 0.69 | 0.66 | 0.75 | 0.68 | 0.72 | 0.70 | 0.71 | 0.74 | 0.73 | 0.72 | 0.68 |
| 0.72 | 0.75 | 0.68 | 0.68 | 0.73 | 0.70 | 0.69 | 0.67 | 0.66 | 0.63 | 0.65 | 0.60 | 0.71 | 0.70 | 0.68 | 0.69 | 0.65 |
| 0.71 | 0.65 | 0.68 | 0.62 | 0.68 | 0.66 | 0.63 | 0.61 | 0.65 | 0.64 | 0.64 | 0.63 | 0.68 | 0.71 | 0.67 | 0.65 | 0.63 |
| 0.72 | 0.74 | 0.70 | 0.68 | 0.73 | 0.72 | 0.68 | 0.67 | 0.70 | 0.63 | 0.69 | 0.65 | 0.73 | 0.74 | 0.71 | 0.73 | 0.67 |
| 0.77 | 0.77 | 0.74 | 0.70 | 0.75 | 0.77 | 0.72 | 0.70 | 0.77 | 0.71 | 0.76 | 0.70 | 0.76 | 0.79 | 0.76 | 0.76 | 0.73 |
| 0.68 | 0.73 | 0.66 | 0.66 | 0.73 | 0.70 | 0.67 | 0.66 | 0.68 | 0.62 | 0.68 | 0.65 | 0.74 | 0.73 | 0.69 | 0.71 | 0.68 |
| 0.74 | 0.74 | 0.70 | 0.70 | 0.73 | 0.70 | 0.69 | 0.67 | 0.72 | 0.66 | 0.63 | 0.64 | 0.71 | 0.72 | 0.70 | 0.71 | 0.65 |
| 0.74 | 0.71 | 0.72 | 0.68 | 0.69 | 0.70 | 0.65 | 0.66 | 0.68 | 0.60 | 0.67 | 0.61 | 0.69 | 0.66 | 0.66 | 0.71 | 0.67 |
| 0.68 | 0.72 | 0.68 | 0.66 | 0.71 | 0.72 | 0.69 | 0.64 | 0.68 | 0.64 | 0.69 | 0.63 | 0.71 | 0.68 | 0.68 | 0.67 | 0.67 |
| 0.62 | 0.63 | 0.64 | 0.60 | 0.65 | 0.62 | 0.63 | 0.58 | 0.63 | 0.64 | 0.70 | 0.63 | 0.68 | 0.66 | 0.63 | 0.63 | 0.61 |

**Supplementary file 1** (cont.): Jaccard dissimilarity index calculated by Darwin for 64 *Brassica* accessions

| Conquest | A04-73NA | Da vinci | Galielo | Exocet | Exagone | Ibiza | Lorenz | Favorite | Billy | Vision | Goya | Verona | Diffusion | Cult | Largo | Prisma | JSv 01-131 | JSv 01-114 | JSv 01-114 | JSv 00-155 | JSv 00-134 | Tianyou-4 | Tianyou-7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.23 | | | | | | | | | | | | | | | | | | | | | | | |
| 0.43 | 0.47 | | | | | | | | | | | | | | | | | | | | | | |
| 0.39 | 0.44 | 0.29 | | | | | | | | | | | | | | | | | | | | | |
| 0.40 | 0.47 | 0.26 | 0.25 | | | | | | | | | | | | | | | | | | | | |
| 0.43 | 0.44 | 0.18 | 0.25 | 0.19 | | | | | | | | | | | | | | | | | | | |
| 0.38 | 0.45 | 0.36 | 0.32 | 0.29 | 0.29 | | | | | | | | | | | | | | | | | | |
| 0.47 | 0.50 | 0.36 | 0.42 | 0.41 | 0.40 | 0.26 | | | | | | | | | | | | | | | | | |
| 0.40 | 0.42 | 0.35 | 0.38 | 0.31 | 0.31 | 0.23 | 0.31 | | | | | | | | | | | | | | | | |
| 0.34 | 0.41 | 0.31 | 0.30 | 0.33 | 0.32 | 0.19 | 0.29 | 0.16 | | | | | | | | | | | | | | | |
| 0.40 | 0.43 | 0.36 | 0.39 | 0.36 | 0.34 | 0.18 | 0.31 | 0.23 | 0.19 | | | | | | | | | | | | | | |
| 0.34 | 0.41 | 0.36 | 0.35 | 0.34 | 0.39 | 0.24 | 0.29 | 0.26 | 0.19 | 0.24 | | | | | | | | | | | | | |
| 0.36 | 0.40 | 0.35 | 0.32 | 0.42 | 0.41 | 0.33 | 0.36 | 0.32 | 0.28 | 0.35 | 0.26 | | | | | | | | | | | | |
| 0.43 | 0.52 | 0.39 | 0.38 | 0.39 | 0.33 | 0.41 | 0.42 | 0.38 | 0.36 | 0.41 | 0.37 | 0.38 | | | | | | | | | | | |
| 0.36 | 0.47 | 0.38 | 0.36 | 0.38 | 0.43 | 0.33 | 0.31 | 0.37 | 0.35 | 0.38 | 0.28 | 0.35 | 0.36 | | | | | | | | | | |
| 0.70 | 0.66 | 0.71 | 0.66 | 0.67 | 0.69 | 0.69 | 0.68 | 0.62 | 0.64 | 0.65 | 0.68 | 0.70 | 0.71 | 0.65 | | | | | | | | | |
| 0.60 | 0.57 | 0.67 | 0.66 | 0.64 | 0.69 | 0.68 | 0.70 | 0.59 | 0.65 | 0.64 | 0.67 | 0.67 | 0.69 | 0.64 | 0.47 | | | | | | | | |
| 0.61 | 0.60 | 0.64 | 0.60 | 0.59 | 0.63 | 0.65 | 0.67 | 0.59 | 0.60 | 0.63 | 0.66 | 0.64 | 0.63 | 0.61 | 0.33 | 0.28 | | | | | | | |
| 0.64 | 0.59 | 0.69 | 0.66 | 0.66 | 0.69 | 0.68 | 0.66 | 0.59 | 0.64 | 0.66 | 0.67 | 0.65 | 0.66 | 0.62 | 0.39 | 0.26 | 0.29 | | | | | | |
| 0.67 | 0.68 | 0.72 | 0.71 | 0.71 | 0.73 | 0.71 | 0.68 | 0.64 | 0.69 | 0.69 | 0.72 | 0.68 | 0.67 | 0.67 | 0.47 | 0.40 | 0.36 | 0.31 | | | | | |
| 0.63 | 0.60 | 0.69 | 0.64 | 0.67 | 0.69 | 0.65 | 0.68 | 0.61 | 0.64 | 0.63 | 0.68 | 0.64 | 0.67 | 0.65 | 0.48 | 0.32 | 0.38 | 0.17 | 0.33 | | | | |
| 0.64 | 0.59 | 0.69 | 0.66 | 0.62 | 0.66 | 0.68 | 0.70 | 0.63 | 0.68 | 0.64 | 0.67 | 0.68 | 0.62 | 0.66 | 0.46 | 0.33 | 0.36 | 0.27 | 0.39 | 0.33 | | | |
| 0.61 | 0.60 | 0.65 | 0.66 | 0.63 | 0.67 | 0.65 | 0.66 | 0.61 | 0.68 | 0.61 | 0.68 | 0.66 | 0.63 | 0.59 | 0.48 | 0.28 | 0.38 | 0.30 | 0.41 | 0.32 | 0.26 | | |
| 0.63 | 0.60 | 0.69 | 0.68 | 0.69 | 0.69 | 0.67 | 0.66 | 0.62 | 0.66 | 0.65 | 0.68 | 0.64 | 0.67 | 0.67 | 0.47 | 0.37 | 0.43 | 0.35 | 0.50 | 0.33 | 0.39 | 0.25 | |
| 0.57 | 0.59 | 0.68 | 0.67 | 0.65 | 0.69 | 0.65 | 0.69 | 0.62 | 0.62 | 0.63 | 0.64 | 0.64 | 0.67 | 0.63 | 0.50 | 0.47 | 0.49 | 0.46 | 0.56 | 0.48 | 0.55 | 0.45 | 0.32 |

## 5. General Discussion

The overall goal of the work presented in this dissertation was to identify cold-responsive miRNAs in canola and to functionally characterize the role of miRNAs in response to CS. To achieve these objectives, canola (spring accession DH12075) plants were subjected to cold stress (CS) treatment and changes to various physiological parameters resulting from stress were measured. Increased electrolyte leakage (EL), higher accumulation of Malondialdehyde (MDA), elevated levels of antioxidant enzymes, decreased chlorophyll and carotenoid content and reduced photosynthetic rate were observed in plants after CS. Although 'DH12075' is a spring canola (with a lower frost tolerance when compared to winter types), it is not sensitive to chilling temperatures. In spite of an increase in EL and MDA levels due to membrane damage after CS, a concomitant increase in activities of antioxidant enzymes (such as POD and CAT) might be indicative of an involvement of redox homeostasis, playing a role in ameliorating the oxidative stress caused by cold.

In an effort to identify cold-responsive miRNAs in *B. napus*, small RNA transcriptome sequencing from control plants and those subjected to CS for different time points resulted in the identification of 70 known and 126 novel miRNAs. Among these, 25 known and 104 novel miRNAs were observed to be differentially expressed (DE) in response to the imposed CS. Consistent with other studies in different plant species cold-responsive miRNAs, including miR394, miR395 and miR397 were identified in this study as well. A total of 252 putative target genes were identified for the aforementioned 129 miRNAs which were differentially expressed in response to CS. In addition, it was

observed that psRNATarget predicted just one *APS1* gene as the target of miR395, but upon manual search of pSRNATarget, the miR395 target site was found to be present in *APS3, APS4* and *SULTR2;1* as well. Thus, the results from such bioinformatics tools must be interpreted carefully. In plants, conserved miRNAs are known to regulate the expression of homologous targets (Axtell and Bowman, 2008) and in this study also conserved miRNAs were found to target members of same TF or protein family. For instance, miR164 targets *NAC* (for *NAM/ATAF1, 2/CUC2)*, miR166 targets members of HD-ZIP III family, miR393 targets F-box genes encoding auxin receptors and miR395 targets ATP sulfurylases (*APS1*, *APS3* and *APS4*). These targets are also conserved among different plant species such as, *Arabidopsis,* rice, populus, tea and tomato (Guo *et al.* 2005; Wu *et al.* 2009; Cao *et al.* 2014; Zhang *et al.* 2014). On the other hand, most of the novel miRNAs showed regulation of diverse array of targets. For instance, putative targets of bna-N_miR2 ranged from, NAM TF (No apical meristem), TRIPTY TF, a sugar transporter, Acyl-CoA binding protein and Cysteine synthase. It has been reported previously that transition of a novel miRNA to conserved one depends on its integration into an indispensable genetic network (Axtell and Bowman, 2008). The regulation of miscellaneous targets by novel miRNAs might be an indication of low frequency of their transition to a conserved miRNA. In addition, no targets were predicted for seven novel miRNAs using psRNA target. The absence of targets can be correlated to the transition from novel to conserved miRNA. As mentioned earlier, the loss or preservation of novel miRNA gene depends on its selective advantage. Most of the times, novel miRNA genes accumulate mutations thus becoming non-functional or they drift so apart that they do not have any interaction with their target

transcripts (Fahlgren *et al.* 2007). Thus, the seven novel miRNAs identified in our study might have accumulated mutations and have no interaction with targets.

Furthermore, as spring and winter canola demonstrates differential tolerance to frost, we speculated that their response to CS would be different. Therefore, in an attempt to assess these differences, we exposed a winter variety of canola 'Mendel' to same CS conditions as 'DH12075'. The expression levels of eight miRNAs and 12 target genes were determined by qRT-PCR in both 'DH12075' and 'Mendel'. The expression pattern of six out of eight miRNAs exhibited an opposite trend in 'DH12075' and 'Mendel' *i.e.* decreased expression in cold-stressed 'DH12075', whereas the expression was induced in 'Mendel. Similarly, the expression of targets of selected miRNAs, such as Auxin signalling F_box transcript (miR393) and Laccase like multicopper oxidase (LLMO) (miR397) decreased in 'DH12075' and increased in 'Mendel' after CS. Differential expression pattern of miRNAs and their target genes in lines with different cold sensitivities has been reported previously in tomato and tea (Zhang *et al.* 2014; Koc *et al.* 2015). These findings suggest that miRNA expression levels may vary in lines in response to CS, depending on their level of tolerance. Owing to these results, it would be interesting to compare the global miRNA changes after CS in spring 'DH12075' and winter 'Mendel'. These additional studies would serve to complement the present work and may provide more clues to the basis of cold tolerance in canola.

It is known that the presence of *cis*-elements in the promoter region largely regulates gene expression level (Hernandez-Garcia and Finer, 2014) therefore; we analyzed the 1.5 kb region upstream of 14 precursor miRNAs for presence of *cis*-elements. The

investigation revealed that abscisic acid (ABA) response element (ABRE) was present in upstream region of all 14 miRNAs. It is known that response to CS affects more than 2000 genes and only 4-20 % of them are regulated by *CBF* regulon (Hannah *et al.* 2005; Lee *et al.* 2005). Thus, it might be speculated that presence of ABA binding sites in cold responsive miRNA genes provides another layer of regulation of CS response. Furthermore, *cis*-elements such as *RAV1*, MYC and LTRE repeat are found in promoter region of low temperature responsive TFs and genes. The presence of such *cis*-elements in the upstream region of miRNAs indicates that these miRNAs might play an important role in regulating plant response to CS.

Next-generation sequencing (NGS) of cold-stressed canola tissues revealed that three members of miR395 family (miR395c, e and 395f) were differentially expressed. Although the expression of miR395 as detected by qRT-PCR showed an up-regulation after CS, the expression patterns for individual miRNA395 members detected by NGS were different. The expression of miR395c varied between different time points, whereas, miR395e and miR395f showed a trend. While miR395e was up-regulated over the course of 0 h-48 h after CS; miR395f expression showed a down-regulation after CS. As mentioned above, members of miR395 family target ATP sulfurylase genes (*APS1*, *APS3* and *APS4*) and the low affinity sulfate transporter gene, *SULTR2;1* (Kawashima *et al.* 2009; Huang *et al.* 2010) are targets of miR395 family. Previous studies have shown that increased expression of *APS* gene can be linked to enhanced cold tolerance via accumulation of antioxidant-glutathione (Kocsy *et al.* 2004; Wang *et al.* 2016). Therefore, it was hypothesized that miR395 might participate in plant defense response against CS and

over-expression of miR395 in *Arabidopsis* might alter CS tolerance. We employed a transgenic approach, through heterologous over-expression of the *bna* pre-miR395f and miR395e in *Arabidopsis* to characterize their potential role in CS regulation. However, we were able to obtain only the pre-miR395f transgenic plants in a timely manner and hence proceeded with testing the *bna* pre-miR395f lines for their cold tolerance. Despite observing an increase of pre-miR395f levels, we were not able to see an increase in the levels of mature miRNA in transgenic lines. Therefore, it can be speculated that pre-miR395f might be interacting directly with the target genes, thereby conferring the observed response towards CS. The direct role of pre-miR395f in regulating target genes can be validated in the future by generating expression cassettes with edited pre-miR395f. In many plant precursors, a single change in the lower stem of 15 nt below the miRNA results in inaccurate and inefficient processing of the precursor due to loss of interaction with key factors promoting miRNA biogenesis (Cuperus *et al*. 2010; Werner *et al.* 2010; Bologna *et al.*2012). Thus, evaluating the effect of mutating the nucleotides near the lower stem of pre-miR395f on precursor and targets abundance can provide evidence on the regulatory role of precursor sequence.

Our results indicate that heterologous expression of *bna* pre-miR395f in *A. thaliana* increased the sensitivity of plants to both cold and freezing temperature. In addition, we observed an increased expression of genes related to sulfur metabolism and those encoding antioxidant enzymes as evidenced by qRT-PCR. Our studies clearly establish that there is a cross talk between different components of the antioxidant pathway and sulphur

homeostasis in CS response, which might be mediated through miR395f. Nevertheless, the specific role(s) of miR395f in mediating CS responses still remains unknown at this time.

Although, the expression of transcripts related to sulfur metabolism and antioxidant enzymes also increased after CS in miR395f-KO compared to WT, there was no increase in the cold tolerance of miR395f-KO. On the contrary, the performance of miR395f-KO under freezing and CS conditions was similar to WT and VC. These differences can be possibly partially explained by the redundancy of miR395 family. For instance, knock out studies on three membered miR164 family revealed that the loss of a single miRNA of this multigene family did not result in an aberrant phenotype (Sieber *et al.* 2007). Thus, to obtain a clear picture of the role of miR395 family in regulating response to CS, complete knockout lines of this family needs to be generated and tested. Additionally, it has been reported previously that OE of miR395e or miR395c (with similar sequence to miR395f) in *A. thaliana* retarded and accelerated, respectively, seed germination under salinity and dehydration stress conditions (Kim *et al.* 2010). Given that miR395f OE lines showed hypersensitivity to CS, it would be interesting to see if the OE of miR395e (differs from miR395f in just one nucleotide) results in increased cold tolerance. Results presented in this dissertation may form the basis for further analysis of the roles of individual members of the miR395 family in mediating CS responses in plants.

As evidenced by work in this dissertation and as well as other studies in the literature, miRNAs play a crucial role in regulating plant's response to CS. Based on the functional role of miRNAs, we initiated a study to determine whether presence of polymorphisms such as SSRs in the miRNA genes can be used to determine genetic

variability between different *Brassica* accessions lines belonging to winter and spring growth habit types. Twenty-five miR-SSRs designed from 90 known *B. napus* miRNA coding genes were used for genetic diversity analysis. These miR-SSR markers were able to distinguish the *Brassica* lines into five different clusters based on their taxonomic classification and growth habit. Although all the 25 miR-SSRs were polymorphic only the marker miR159-SSR was able to distinguish winter and spring growth habit *Brassica* accessions indicating its potential association with cold tolerance. This polymorphism can be linked to either differential regulation or processing of pre-miRNA from miRNA gene and thus culminating in a differential expression of mature miRNA. In future, the expression profile of mature miR159 can be determined in *Brassica* accessions exposed to freezing temperatures. This can further help in determining the potential association of miR159-SSR to cold tolerance. The pre-miRNA sequences used in this study were from *B. napus*, it would be a good idea to also include pre-miRNA sequences *B. rapa* in future studies which can increase the number of markers used. As development of highly efficient and stable molecular markers is crucial for molecular genetics research and molecular breeding, in this study we developed 25 such molecular markers from *MIRNA* genes. The development of such highly polymorphic miRNA-based molecular markers, associated with specific traits, can offer advantages over other marker systems, to plant breeders for crop improvement programs.

In summary, the research described in this dissertation has provided new insights into the miRNAome of cold-stressed canola and further demonstrated that there are contrasting patterns for the selected miRNAs and their targets in cultivars with differential tolerance to CS. In addition, results presented in this dissertation suggest that the

heterologous expression of *bna* pre-miR395f in *A. thaliana* increases the cold sensitivity through modulation of sulphur metabolism, antioxidant levels and enzymes; and demonstrated that miRNA-SSR markers could be employed as molecular markers to differentiate *Brassica* lines based on their resistance to CS. Future studies may be aimed at understanding the role of miR395 family members in regulating CS response by generating transgenic plants over-expressing different members and by complete knockout of miR395 family.

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## **Appendix:** Response of transgenic plants to salt stress conditions

### A1: Introduction

Salt stress is a serious abiotic stress of crop plants worldwide affecting ≈ 20 % of irrigated land (Qadir *et al.* 2014). Similar to CS, exposure of plants to salt results in changes in various physiological and metabolic processes, such as ion homeostasis, synthesis of osmoprotectants, compatible solutes, polyamines and antioxidant compounds, depending on severity and duration of the stress (Munns, 2005; Rozema and Fowlers, 2008). At the transcriptional level, a large number of genes are induced in response to salinity in different plant species (Gupta and Huang 2014). Senescence-associated genes (*SAG*), dehydration-related TFs (*DREB*), ion transport or homeostasis related genes (*SOS* genes, *AtNHX1*, and $H^+$-*ATPase*) are some of the key players involved in response to salt stress (Gupta and Huang, 2014). In addition, a number of miRNAs showing differential expression in response to salt stress have been identified in various plant species (reviewed in Shriram *et al.* 2016). For instance, differential expression patterns for miR156, mir159, miR156, miR164 and miR167 have been reported in rice, wheat and barley under salinity stress (Zhang 2015; Shriram *et al.* 2016). Interestingly, in rice and wheat, while the expression of miR159, miR393, miR399 was both up-regulated under salinity and CS the expression of miR169, miR394 and miR396 was up-regulated under salinity stress and down-regulated under CS conditions (reviewed in Zhang 2015). Clearly, a cross talk occurs between cold and salinity stresses with respect to the expression of miRNAs. Thus, an attempt was made to determine the effect of miR395f over-expression on salt stress tolerance.

## A2: Material and Methods

In this study, WT, VC, transgenic lines and 395-KO was used and all the lines were in the Columbia ecotype. Seeds were sterilized with 30 % bleach solution for 20 min and rinsed thoroughly with sterilized water for four times. The sterilized seeds were germinated on ½ Murashige and Skoog (MS) medium with 1.5% sucrose and 0.7 % phytoagar (pH=5.7). For germination experiments, seeds were grown on ½ MS supplemented with 125mM NaCl. Germination count of seeds was recorded every day for five days and those seeds in which the radicle had emerged were considered to have germinated. Endogenous Chl-*a,* Chl-*b* and carotenoid contents were determined as described in Chapter 2. The expression of genes related to sulfur-metabolic pathways was quantified from three week old seedlings grown on 0 mM (control) and 125 mM NaCl. The data were statistically analysed by means of one-way analysis of variance (ANOVA) with post hoc comparisons using Dunnett t- test in SPSS, taking $P < 0.05$ as significant.

## A3: Results and Discussion

No phenotypic differences were observed when transgenic plants plants (OE#3.5, OE#4.4 and OE#6.8), WT, VC and miR395f-KO were grown under control conditions (Figure A1). The percent reduction (salt / control) in Chl-*a*, Chl-*b* and carotenoid contents were measured. As shown in Fig. A2, Chl-*a* content increased under salt stress in all the lines tested. In two out of three transgenic lines (#3.5 and #6.8) Chl-*a* content increased significantly compared to WT (Figure A2). Furthermore, content of Chl-*b* decreased in all the lines tested under salt stress as indicated by reduction of 46 % to 85 % (Figure A2).

The Chl-*b* content of only one transgenic lines (#6.8) and miR395f-KO was significantly increased compared to WT. Reduction in photosynthetic pigments, such as Chl-a and

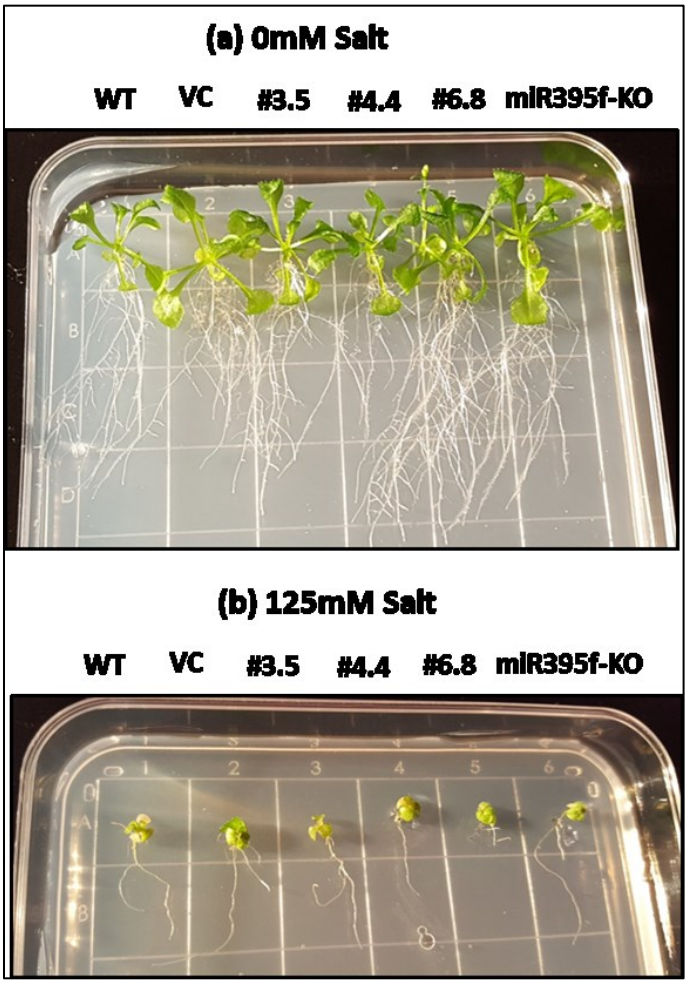


**Figure** A1: **Effect of salinity on the seedlings of WT, VC, OE#3.5, OE#4.4, OE#6.8 and miR395f-KO germinated and grown on 0mM salt (a) and 125 mM salt (b).**

Chl-b has been reported in some studies in different crops, e.g., *Heliantus annuus* (Akram and Ashraf 2011), *Triticum aestivum* (Perveen *et al.* 2010), and *Brassica juncea* (Pandey and Penna, 2016). However, during the process of Chl degradation, Chl-b is converted into Chl-a, thus resulting in the increased content of Chl-a (Hotensteiner and Krautlerm 2011).

While under salt stress, higher carotenoid levels were observed in all lines tested except #4.4 (Figure A2), the carotenoid levels were significantly reduced in #3.5 and #6.8, compared to WT (Figure A2). Lower carotenoid levels may contribute to the decreased tolerance of transgenic lines towards salt stress.

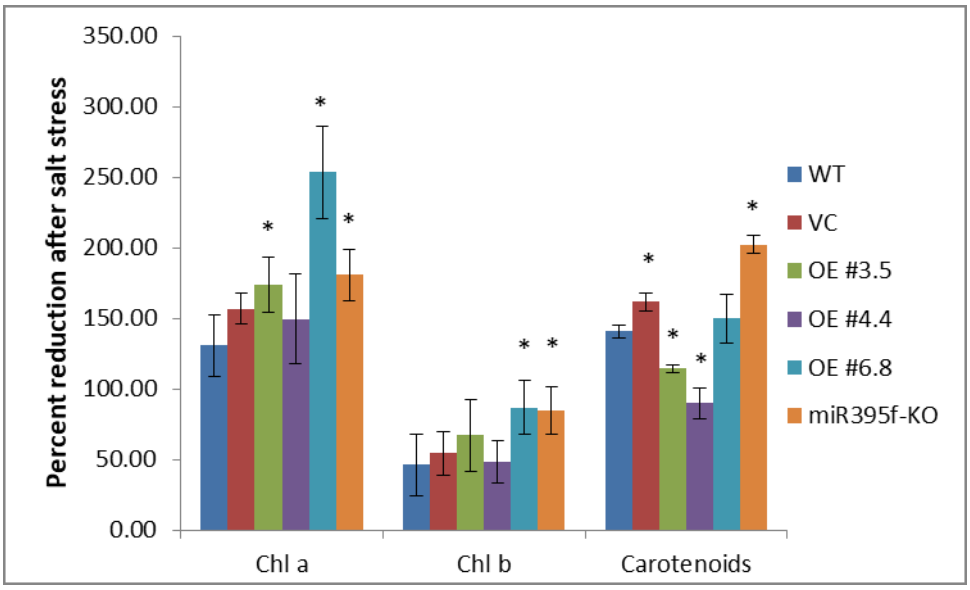


**Figure A2**: **Percentage reduction in Chlorophyll a, b and carotenoid contents after salt stress of 125mM in WT, VC, OE#3.5, OE#4.4, OE#6.8 and miR395f-KO.**

Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic lines is significantly different from WT ($P$ <0.05).

Seed germination and post-germination growth of miR395f-OE *Arabidopsis* under 0 mM and 125 mM NaCl was determined over five days. The percent germination was significantly reduced as compared to WT in all three transgenic lines on day 3 (Figure A3). No significant differences in seed germination were observed among all lines tested on other days (Figure A3). Greening of cotyledons was also reduced significantly in

transgenic lines compared to WT on day 2 and 3. As shown in Figure A3, seed germination of transgenic lines was significantly reduced from day 2 to day 4 compared to WT under salt stress, while no significant differences were found at day 5 between transgenic lines and WT. Similarly, on day 2, the cotyledon greening of transgenic lines was only 11-15 %, whereas that of WT was 27 % (Figure A3). Reduced seed germination and seedling growth has been previously reported in *Arabidopsis* plants over-expressing miR395c, although no significant differences in seed germination under salt stress were observed (Kim *et al.* 2010). These results demonstrate that miR395f has negative effect on seed germination under salt stress.

Transcript levels of target mRNAs (*APS1, APS3, APS4, SULTR2:1*) were determined after three week of salt stress (Figure A4). No significant differences were observed in the expression level of *APS1* and *APS3* in transgenic plants compared with WT; while the expression of *APS4* and SULTR2*;1* reduced significantly in all three transgenic lines (Figure A4). It can be speculated that down-regulation of *APS4* and *SULTR2;1* expression in transgenic plants results in decreased sulfate assimilation and transport which leads to reduced seed germination and growth, a hypothesis which remains to be tested.

Changes in expression level of transcripts of 18 enzymes related to sulfur metabolism as well as for antioxidant enzymes (measured in Chapter 3) were also determined in control and salt stressed plants. We were not able to quantify the changes in expression of *Serate3;2* transcript under salt stress and hence it was excluded from the results. Out of the remaining 17 transcripts, only five showed significant differential

expression in transgenic lines compared to WT under salt stress (Figure A5). While, the expression of *GPX1, GPX2* and *GPX4* showed a significant increase in transgenic lines compared to WT plants; the expression of *GPX3* decreased in transgenics under salt stress (Figure A5). Previously, the transcript levels of *GPX1, GPX2, GPX5* and *GPX6* were found to increase after salt stress in *A. thaliana*, while the expression of other members remained steady (Milla *et al.* 2003). Thus, the expression of *GPXs* is differentially regulated under salt stress. Further work is needed to better understand the role of miR395f and other members of miR395 family in regulating the response towards salt stress.

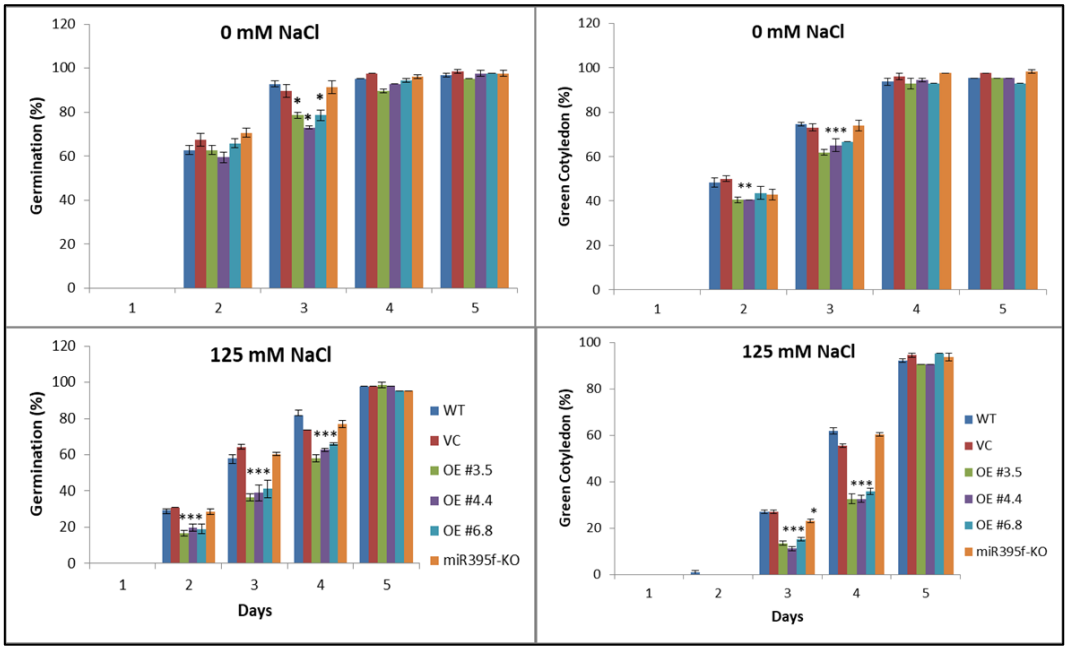


**Figure A3**: **Germination and growth response of WT, VC, OE#3.5, OE#4.4, OE#6.8 and miR395f-KO plants to salt stress. Error bars indicate SE (n=3).**

Asterisks indicate that expression in transgenic line is significantly different from WT ($P$ <0.05).

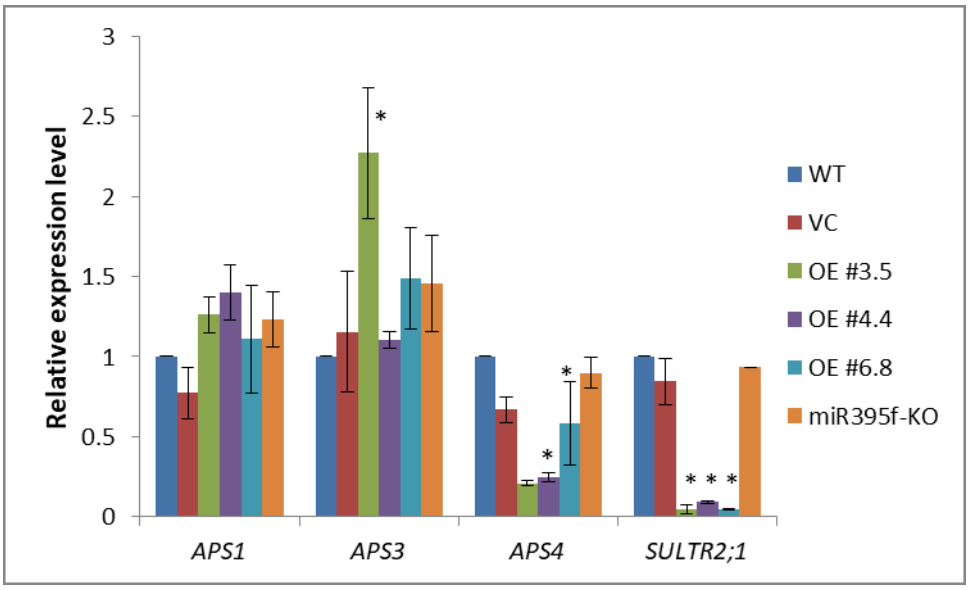


**Figure A4**: **Expression levels of miR395f target mRNAs under salt stress. Error bars indicate SE (n=3).**

Asterisks indicate that expression in transgenic line is significantly different from WT ($P$ <0.05).

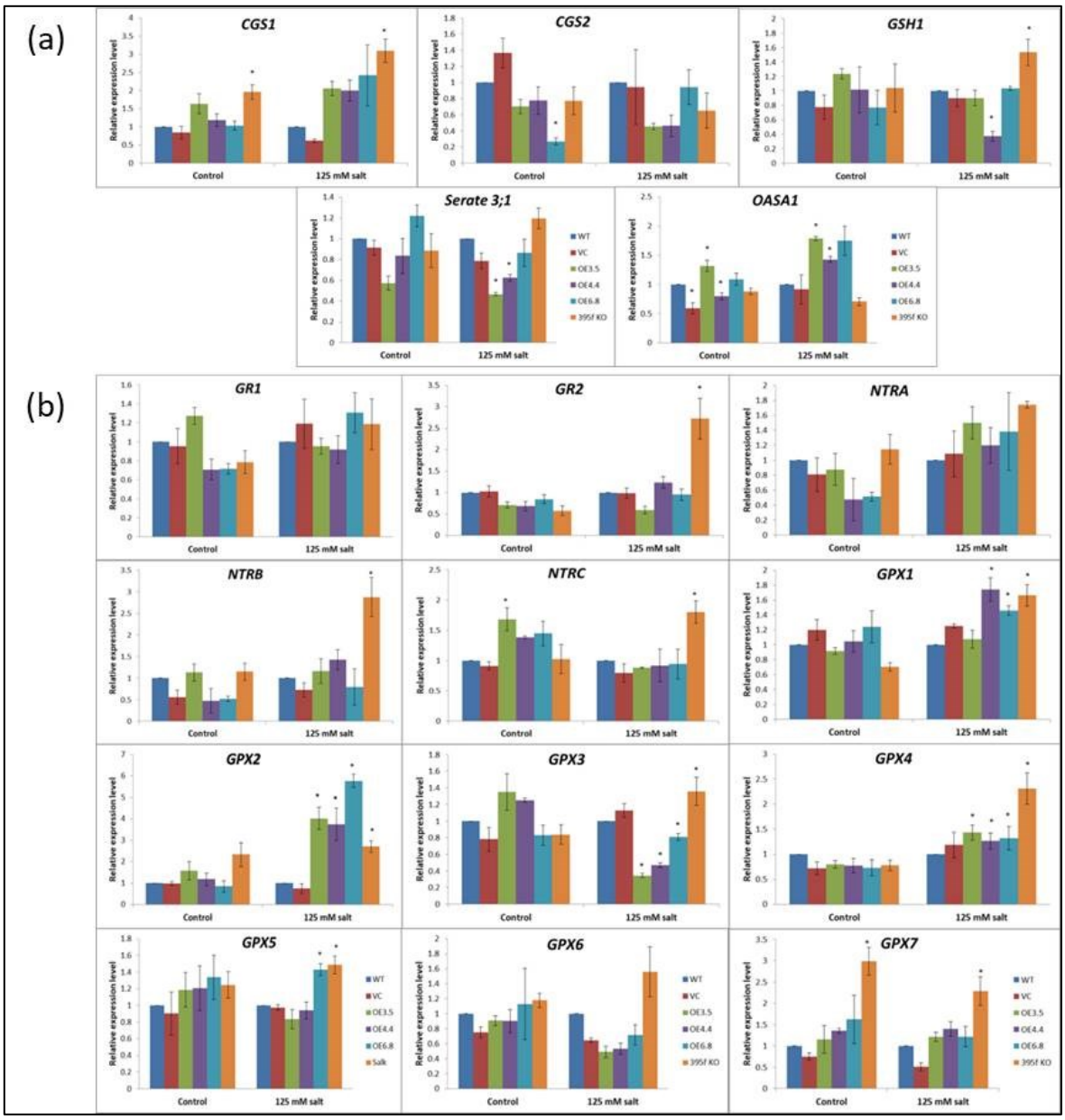


**Figure A5**: **Changes in expression level of transcripts related to sulfur metabolism in seedlings grown under 0 and 125 mM salt.**

Error bars indicate SE (n=3). Asterisks indicate that expression in transgenic line is significantly different from WT ($P$ <0.05).

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