Title**: Regulation of low temperature stress in plants by microRNAs**

Running title: **Role of miRNAs in low temperature response**

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Abstract

Low temperature is one of the most common environmental stresses that seriously affects the growth and development of plants. However, plants have the plasticity in their defense mechanisms enabling them to tolerate and, sometimes, even survive adverse environmental conditions. MicroRNAs (miRNAs) are small non-coding RNAs, approximately 19-21 nucleotides in length, and are being increasingly recognized as regulators of gene expression at the post-transcriptional level and have the ability to influence a broad range of biological processes. There is growing evidence in the literature that reprogramming of gene expression mediated through miRNAs, is a major defense mechanism in plants enabling them to respond to stresses. To date, numerous studies have established the importance of miRNA-based regulation of gene expression under low temperature stress. Individual miRNAs can modulate the expression of multiple mRNA targets and, therefore, the manipulation of a single miRNA has the potential to affect multiple biological processes. Numerous functional studies have attempted to identify the miRNA-target interactions and have elaborated the role of several miRNAs in cold-stress regulation. This review summarizes the current understanding of miRNA-mediated modulation of the expression of key genes as well as genetic and regulatory pathways, involved in low temperature stress responses in plants.

Keyword Index: MicroRNAs, low temperature, cold stress, CBFs, cold-acclimation

Introduction

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 (< 4°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 *et al.* 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).

The purpose of this article is to review available literature on miRNAs and their role in mediating plant responses to 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.

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). 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 *Arabidopsis*, *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 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 *Arabidopsis* resulted in plants with low electrolyte leakage and less accumulated Malondialdheyde (MDA), and thus cold-tolerant plants (Zhu *et al.* 2010). Moreover, Hsp expression is induced by cold stress 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 PR (pathogen-related) proteins, such as PR1, PR2 (β-1,3-glucanase) and PR5 (thaumatin-like proteins) have been found to have antifreeze properties (Venketesh & Dayananda 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). *Arabidopsis* 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 & Parish, 2008). All these studies clearly establish the important role of different cold-regulated genes and their products in modulation of the cold stress response.

In *Arabidopsis*, 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 *Arabidopsis* (Van-Buskirk and Thomashow 2006; Chinnusamy *et al.* 2007). Followed by their discovery and functional characterization in *Arabidopsis*, CBF homologs have been identified in a variety of monocots and dicots, including rice, wheat, barley, and *Brassica 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

114 *et al.* 2003; 2007; Fursova *et al.* 2009; Doherty *et al.* 2009; Kim *et al.* 2013). *ICE1* encodes a
115 bHLH (basic helix-loop helix) protein, a constitutive TF, which gets activated at low temperature
116 and acts upstream of the *CBF3* in cold-responsive pathways (Chinnusamy *et al.* 2003; Zarka *et*
117 *al.* 2003; Lee *et al.* 2005) (Figure 1). Overexpression of *ICE1* and *ICE2* in transgenic plants has
118 been shown to increase the expression of *CBF3* and *CBF2* (Chinnumsamy *et al.* 2003; Fursova *et*
119 *al.* 2009). CAMTA3 binds to *CBF2* promoter resulting in increased expression of *CBF2* under
120 cold stress (Doherty *et al.* 2009). *Arabidopsis* mutants of CAMTA TF have shown decreased
121 ability to cold acclimate, indicating their role in regulation of *CBF* expression (Doherty *et al.*
122 2009; Kim *et al.* 2013). It can be concluded from all these studies that although *CBF* genes have
123 similar biological functions, the regulation of their expression is considerably complex.

124 Overexpression of *CBF* genes enhances the cold tolerance of *B. napus* (Jaglo *et al.* 2001),
125 poplar (Benedict *et al.* 2006), and potato (Pino *et al.* 2007). In *Arabidopsis*, constitutive
126 overexpression of *CBF1* and *CBF3* has been shown to activate the entire cascade of known
127 CBF/DREB regulated *COR* genes, even at warm temperatures, and resulted in enhanced freezing
128 tolerance (Jaglo *et al.* 1998; Gilmour *et al.* 2000). Based on results from transcriptomic and
129 metabolomics studies, it was concluded that the improved stress tolerance of *Arabidopsis* plants
130 overexpressing *CBF1* may be due to an accumulation of various beneficial metabolites and
131 through the induction of many stress-responsive genes (Fowler and Thomashow 2002;
132 Marumyma *et al.* 2004, 2009). However, the constitutive overexpression of *CBF* under the
133 control of the CaMV 35S promoter resulted in a 'stunted' growth phenotype and delayed
134 flowering in *Arabidopsis*, *B. napus,* and rice (Gilmour *et al.* 2000; Jaglo *et al.* 2001; Ito *et al.*
135 2006). The use of stress-inducible rd29A promoter instead of the constitutive promoter for
136 overexpression 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 cold stress tolerance (Kasuga *et al.* 1999; Zhang *et al.* 2009a; Ishizaki *et al.* 2013; Kidokoro *et al.* 2015). In contrast, observations on *Arabidopsis* 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), suggesting 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). 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).

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 *Arabidopsis* 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 & Griffith-Jones 2014). As alluded to previously, miRNAs are non-coding RNA molecules which are 19-24 nt in length and function as gene regulators in diverse organisms. In plants, they 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.* 2011; 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 *Arabidopsis,* rice and wheat (Piriyapongsa and Jordan 2008; Li *et al.* 2011; 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 *Arabidopsis* 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 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 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). The pre-miRNA is further cleaved 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 mature miRNAs, are orchestrated by Dicer Like-1 (DCL1), a type III RNAse which is assisted by the dsRNA binding protein Hypnostic leaves1 (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). 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 *et al*. 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, 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.

MiRNAs responsive to LT stress

MiRNAs were demonstrated to be involved in the regulation of cold stress for the first time by Sunkar and Zhu (2004). Small RNA libraries were constructed from *Arabidopsis* 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 cold stress (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). 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 *Arabidopsis*, poplar and rice from years 2008-2010 (Table 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 cold stress (Pritchard *et al.* 2012). 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).

Differential profiling of LT-induced miRNAs using microarray and next generation sequencing platforms has been reported from various plant species (summarized in Table 1), including *Arabidopsis* (Liu *et al.* 2008), *Populus* (Zhang *et al.* 2009b; Chen *et al.* 2012), rice (Lv *et al.* 2010), *Hemerocallis fulva* (An *et al.* 2014), tomato (Cao *et al.* 2014), grapevine (Sun *et al.* 2015) and almond (Karimi *et al.* 2016). Microarray analysis of LT-treated *Arabidopsis* 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 *Arabidopsis, 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 *Arabidopsis, 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 *Arabidopsis* 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 *Arabidopsis* (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

297 stress (Zhang *et al.* 2014a). An inverse trend was observed for miR474, which was down-
298 regulated in cold-tolerant and up-regulated in cold-sensitive cultivar (Zhang *et al.* 2014a). In
299 wild tomato cultivar 'LA1777' with high chilling tolerance ability, Cao *et al.* (2014) identified
300 192 and 205 miRNAs with increased and decreased expression respectively, after chilling.
301 Despite some variance, similar trends were observed in the expression of six conserved and three
302 novel miRNAs in another chilling tolerant tomato cultivar 'Hezouo908'when subjected to same
303 treatment as LA1777 (Cao *et al.* 2014). Both of these studies suggest that miRNAs may play a
304 cultivar specific role in regulating LT stress tolerance.

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

320 expression of different members of miR156 family (a, b, g, h, i) was down-regulated in both
321 tissue types indicating the possibility that they may share the same regulatory mechanisms in
322 different tissues (Karimi *et al.* 2016). It can be concluded from these observations that same
323 members of miRNAs may show varied or similar expression patterns in different plant tissues.

324 In addition to the aforementioned varying expression patterns of miRNAs observed in
325 different tissues, the duration of LT stress may also alter their expression patterns. For example,
326 the expression pattern of miR398 in grapevine and tomato showed a similar downward trend at
327 varying time points (8h, 24h, 48h) under LT of 4°C; but at the 4h time point, expression of
328 miR398 peaked to a 7-fold change only in grapevine (Cao *et al.* 2014; Sun *et al.* 2015).
329 Similarly, when comparing the expression of miR395 in grapevine and *Populus* over a LT stress
330 period of 2-8h, grapevine miR395 showed a slight increase in expression at 2h, while the
331 expression of *Populus* miR395 decreased at 2h (Chen *et al.* 2012; Sun *et al.* 2015). It has also
332 been observed that the expression of species-specific miRNAs can also be affected by the
333 duration of LT stress. For instance, a tomato specific miRNA, miR69.5p, exhibited higher
334 expression after 1 and 8h of stress, whereas it was observed to be down-regulated after 4, 12, 24
335 and 48h of cold stress (Cao *et al.* 2014). Interestingly, in *Populus,* the expression of cold-
336 responsive pto-miRS16 and pto-miRS16* exhibited inverse patterns, with miRS16* peaking at
337 8h and miRS16* decreasing at same time point (Chen *et al*. 2012). Differential expression of
338 both miRNA and miRNA* suggests involvement of miRNA* in regulating responses to LT.
339 Other recent findings have found a notably high accumulation of miRNA* and subsequent
340 down-regulation of targets (Okamura *et al.* 2008; Yang *et al.* 2011; Devers *et al.* 2011). These
341 observations suggest that there may be additional factors regulating the expression of miRNAs
342 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.

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 *Arabidopsis* and rice (Sunkar *et al.* 2006; Yuzhu *et al.* 2010). Oxidative stress causes down-regulation of miR398 expression both in *Arabidopsis*, 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 2). 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 cold stress 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 2). *Rf-1* is up-regulated under cold stress, 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 *Arabidopsis* has demonstrated that under cold stress, 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 Transcription factor Y, NF-Y), leaf and vascular development (e.g. HD-ZIP proteins, F-box protein), root elongation (e.g. NF-Y) to ROS signalling (e.g. Cu/Zn SODs), and LEA proteins (Figure 3). 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 are potent regulators, which modulate LT responses in different plants by controlling the expression of their target genes.

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; 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 overexpression (OE) in *Arabidopsis* 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 cold stress (-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 cold stress. It is possible that reduced levels of cuproproteins such as cupredoxin in miR408 overexpression lines might be increasing the endogenous availability of copper for other cuproproteins involved in mediating responses to abiotic stress, for example, CSDs (Figure 4). 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 *Arabdiopsis* (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 overexpression and LT responses

In *Arabidopsis*, miR397 exists in two isoforms, miR397a and miR397b, both located on chromosome 4 and differing in only one nucleotide (Sunkar and Zhu 2004). Overexpression of miR397a in *Arabidopsis* 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 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 *Arabidopsis*, 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 4).

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 *Arabidopsis* 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 *Arabidopsis* (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 overexpression of miR394a and *LCR* in *Arabidopsis* 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 *pmiR394a/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 cold stress (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 *Arabidopsis* 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 cold stress 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 5). 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 cold stress 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 *Arabidopsis*, 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 cold stress, 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 cold stress 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. This leads to the up-regulation of *CBF* genes and ROS-scavenging enzymes and increased cold tolerance (Figure 5). 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). Overexpression 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 it's legitimate target 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 5). Since ACO is the rate-limiting enzyme involved in ethylene biosynthesis, a decreased level of ethylene under cold stress 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 cold stress response by

targeting C*BF* pathway (Shi *et al.* 2012; Shi *et al.* 2015); it would be interesting to further elucidate the interplay between ethylene-ACO-miR396b.

Conclusion and future perspectives

MiRNAs are considered to be pivotal factors in determining the specificity of post-transcriptional regulation and gene regulatory networks. Cold acclimation and tolerance are complex processes and involve a number of genes, TFs and miRNAs and, the detailed mechanism of miRNA involvement in LT stress is poorly understood. A combination of studies has provided evidence for miRNAs in orchestrating LT stress responses and has led to the discovery of an entire new layer of gene regulation at transcriptional and post-transcriptional levels. In the present review, we have summarized the biogenesis of miRNAs, and highlighted the role of particular miRNAs and their targets involved in LT stress responses. Six conserved miRNAs discussed in this review have been implicated to control multiple gene networks involved in cold stress. Interestingly, some miRNAs have been implicated in regulation of multiple biological processes and uncovering the miRNA targets for novel and conserved miRNAs, will help in dissecting the molecular regulatory networks in response to LT stress. In addition, the identification of promoter regions of key LT responsive miRNAs, development and characterization of these promoter regions, using gene editing technologies like clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein-9 nuclease (Cas9) may prove promising in devising strategies for improvement and management of crops in response to cold. In addition, the investigation of other layers of regulation of miRNA expression, which may be downstream of transcriptional regulation, may also prove to be valuable towards increasing our understanding of the regulation of miRNA biogenesis. The knowledge on epigenetic mechanisms underlying cold regulation via transcriptional and post-

570 transcriptional means is narrow and more intensive research is needed to fill the gaps in
571 understanding these regulatory processes.

572 **Acknowledgments**

573 Work in the NNVK laboratory is supported in part through a Discovery Grant provided
574 by the Natural Sciences and Engineering Research Council (NSERC) of Canada, which is
575 gratefully acknowledged.

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Figure Legends

Figure 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. microRNAs 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

Figure 2: The target site of *Arabidopsis* miR398a/b/c and rice miR1425 is represented in the figure. The arrows indicate the cleavage sites and localized between the nucleotides 10 and 11 of the miRNA.

Figure 3: Target genes of miRNAs identified by different groups under cold stress 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.

Figure 4: 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

Figure 5: Overview of role of three different miRNAs (from overexpression 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-regulated 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 (represented by green oval) and decreased levels of MDA and ROS activity (represented by red oval).

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

Table 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.* 2009
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* ↓ 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.* 2014
Camellia sinensis; cold tolerant vs. sensitive; 20 day old plants; 4°C; 1, 4, 8, 12, 24, and 48 h	↑ 31, 46* ↓ 43, 45*	6	Zhang *et al.* 2014
Tomato (LA1777) 5 leaf stage seedlings; 1, 4, 8, 12, 24, and 48 h	↑ 12, 11* ↓ 20, 6*	6, 3*	Cao *et al.* 2015
Prunus dulcis Mill; Anther & 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* ↓ 29, 4*	13	Sun *et al.* 2016
Glycine max (Taiwan 75); One-true-leaf stage; 4°C for 24 h	↑ 21, 30*	33, 2*	Xu *et al.* 2016

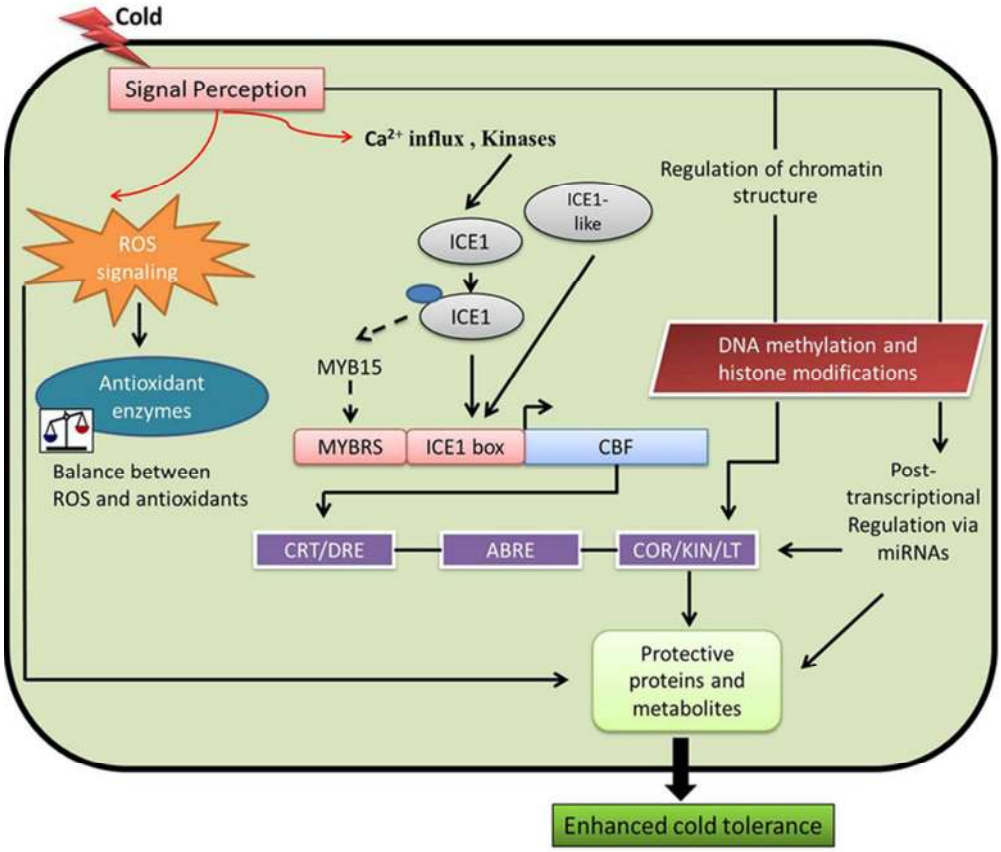

Figure 1

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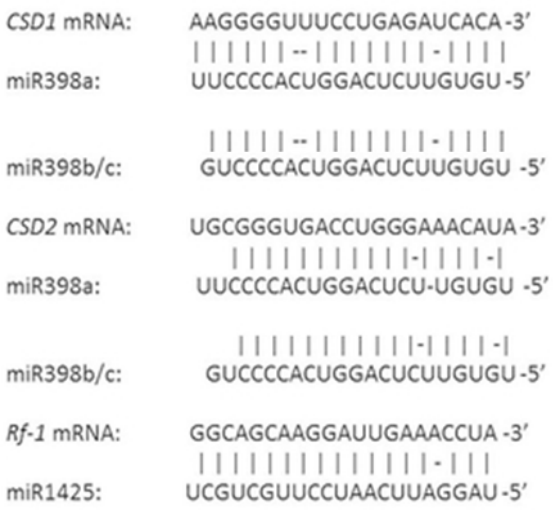

Figure 2

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Figure 3

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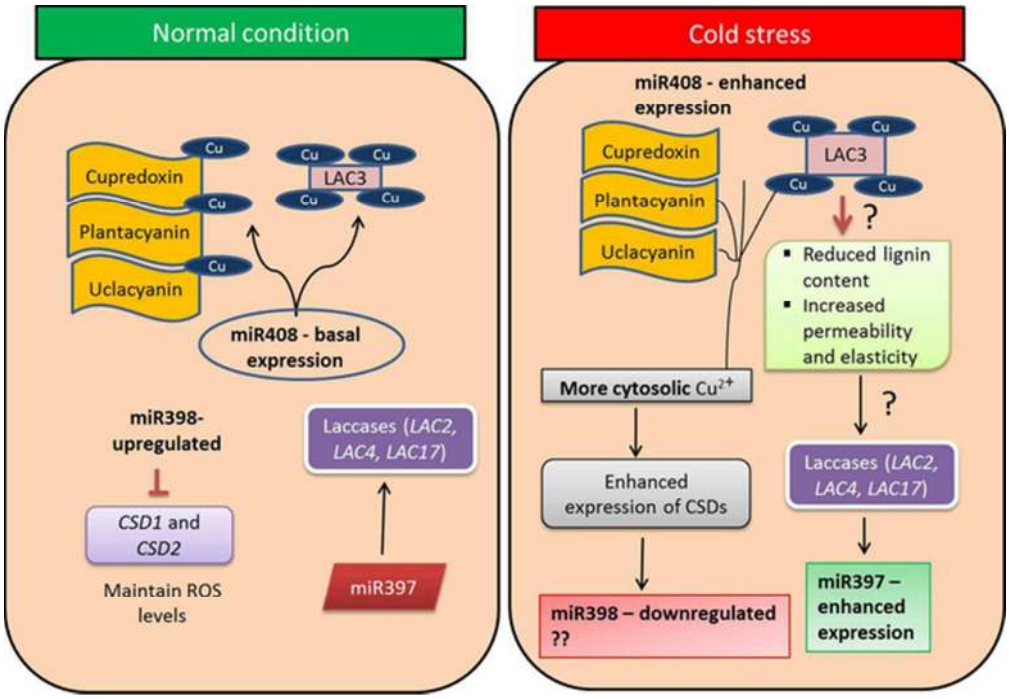

Figure 4

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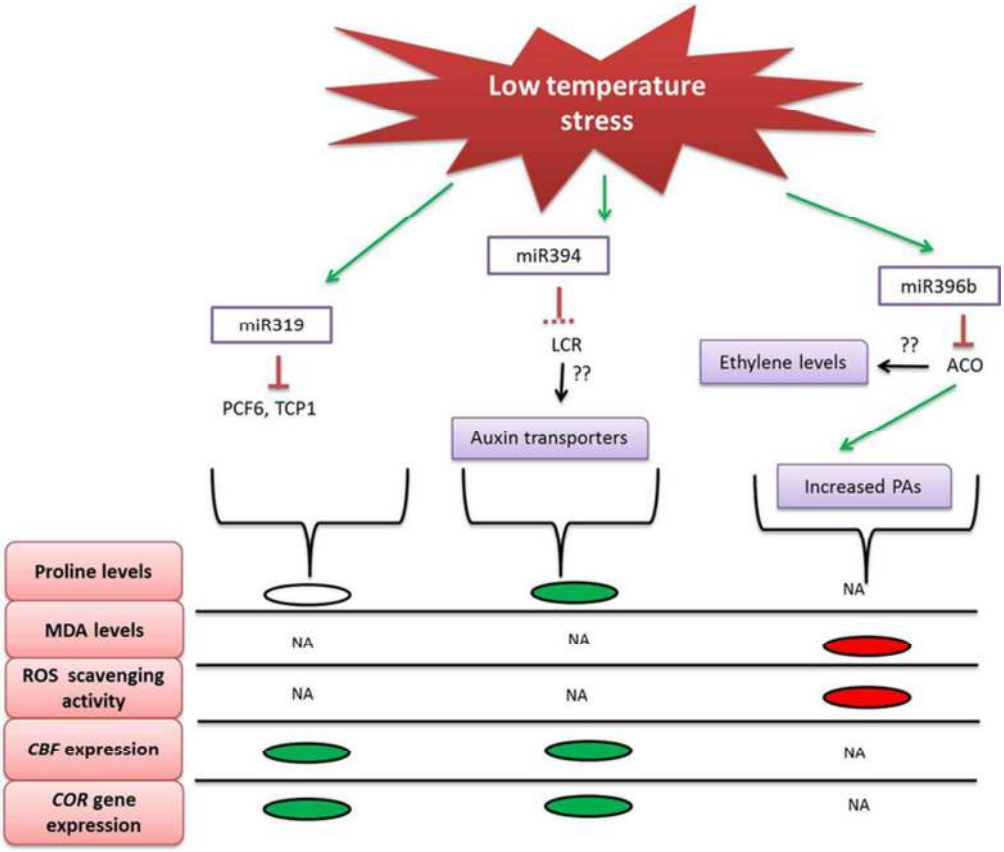

Figure 5

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