



REVIEW PAPER

# MicroRNA: a new target for improving plant tolerance to abiotic stress

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Received 14 December 2014; Revised 14 December 2014; Accepted 17 December 2014

## Abstract

**MicroRNAs (miRNAs) are an extensive class of endogenous, small RNA molecules that sit at the heart of regulating gene expression in multiple developmental and signalling pathways. Recent studies have shown that abiotic stresses induce aberrant expression of many miRNAs, thus suggesting that miRNAs may be a new target for genetically improving plant tolerance to certain stresses. These studies have also shown that miRNAs respond to environmental stresses in a miRNA-, stress-, tissue-, and genotype-dependent manner. During abiotic stress, miRNAs function by regulating target genes within the miRNA–target gene network and by controlling signalling pathways and root development. Generally speaking, stress-induced miRNAs lead to down-regulation of negative regulators of stress tolerance whereas stress-inhibited miRNAs allow the accumulation and function of positive regulators. Currently, the majority of miRNA-based studies have focused on the identification of miRNAs that are responsive to different stress conditions and analysing their expression profile changes during these treatments. This has predominately been accomplished using deep sequencing technologies and other expression analyses, such as quantitative real-time PCR. In the future, more function and expression studies will be necessary in order to elucidate the common miRNA-mediated regulatory mechanisms that underlie tolerance to different abiotic stresses. The use of artificial miRNAs, as well as overexpression and knockout/down of both miRNAs and their targets, will be the best techniques for determining the specific roles of individual miRNAs in response to environmental stresses.**

**Key words:** Abiotic stress, climate change, drought, gene network, microRNA, salinity.

## Introduction

Human population growth and global industrialization are two factors that generate and promote climate change. Both of these factors are exponentially increasing and, therefore, new arable lands for cultivating dedicated food, cash, and biofuel crops will be necessary in order to sustain future generations. However, climate change has the ability to alter atmospheric conditions and modify environmental soils, which can make plant growth and development more difficult. It is well known that environmental abiotic stresses, such as drought and salinity, significantly affect plant survival, growth, and development, and thus decrease plant quality, yield, and biomass production (Wang *et al.*, 2003; Mittler, 2006). The effects of abiotic stresses may also reflect at different suborganismal

levels, including at the biochemical, physiological, cellular, molecular, and even finally at the organismal level. Many studies have demonstrated that abiotic stresses inhibit seed germination, seedling development, root development, chlorophyll biosynthesis, and photosynthesis, and that induced oxidative stresses, such as the production of reactive oxygen species (ROS), further damage plant growth and development (Fernandez, 2014; Mathur *et al.*, 2014; Suzuki *et al.*, 2014). Abiotic stresses have also been shown to alter gene expression profiles significantly during different developmental stages; these gene expression programmes changes ultimately regulate plant developmental and timing plasticity (Fernandez, 2014; Mathur *et al.*, 2014; Suzuki *et al.*, 2014).

Several important genes, including those encoding transcription factors, have been implicated in response to abiotic stresses, and, when these genes were overexpressed in model plant species, such as *Arabidopsis*, as well as other agriculturally important crops, they were shown to improve plant tolerance to individual stresses significantly (Ganesan *et al.*, 2012; Diaz-Vivancos *et al.*, 2013; Liu *et al.*, 2013; Gong *et al.*, 2014; Lee *et al.*, 2014; X. Li *et al.*, 2014; Rong *et al.*, 2014; Tamirisa *et al.*, 2014). Over the past decade, the emergence of new technologies, such as microarrays and next-generation sequencing, has led to the discovery of hundreds of protein-coding genes that are associated with a wide range of environmental abiotic stresses (Baxter *et al.*, 2014; Gollmack *et al.*, 2014). However, there are many questions that remain unanswered, such as how are these protein-coding genes regulated? What kind of gene networks do plants utilize in order to respond to different abiotic stresses? A recently discovered small regulatory RNA molecule, termed microRNA (miRNA), may be the answer to these questions.

miRNAs are an extensive class of small endogenous RNA molecules that range between 20 and 24 nucleotides in length. It is thought that miRNAs are widely distributed throughout the plant kingdom and are highly evolutionarily conserved from mosses to higher flowering monocots and dicots, such as *Arabidopsis*, rice, and cotton (Axtell and Bartel, 2005; Zhang *et al.*, 2006). However, the application of new techniques has led scientists to observe not only that an individual plant species contains these conserved miRNAs, such as miR156, but also that they contain a high number of species-specific miRNAs. This suggests that conserved miRNAs may regulate common traits in plants, such as plant morphology and phase change, and that species-specific miRNAs may control unique and variable processes in individual plant species, such as fibre initiation and development in cotton (Xie *et al.*, 2015a). Both conserved and species-specific miRNAs may be involved in, and play an important role in, plant response to abiotic stress.

In this review, we will first present miRNAs that have been found to respond to different environmental stresses and discuss their expression in a miRNA-, stress-, tissue-, and genotype-dependent manner. Next, we will focus on miRNA-regulated gene networks during plant response to stress. Finally, we will propose the potential application of miRNAs as a new target for genetically improving plant tolerance to abiotic stress. Current existing problems and future directions in this exciting field will also be discussed.

## Abiotic stresses induce the aberrant expression of miRNAs

The role of miRNAs in plant response to abiotic stress was initially suggested after data gathered from miRNA target prediction, miRNA expression profile studies during plant response to abiotic stress, and surveys of NCBI expressed sequence tags (ESTs). In one of the earliest plant miRNA papers, Jones-Rhoades and Bartel (2004) predicted and validated that ATP sulphurylase (APS), the enzyme that

catalyses the first step of inorganic sulphate assimilation, was one of the targets of miR395, which is responsive to sulphate levels in plants. Based on this initial result, they further analysed the response of miR395 to cellular sulphate levels. Their results showed that in comparison with plants growing under normal sulphate conditions (2 mM  $\text{SO}_4^{2-}$ ), miR395 was induced by >100-fold under low sulphate treatment (0.02 mM  $\text{SO}_4^{2-}$ ), suggesting that miR395 is involved in sulphate uptake and metabolism in plants. At the same time, Sunkar and Zhu (2004) constructed small RNA libraries from *Arabidopsis* seedling samples treated with cold stress (0 °C for 24 h), salt stress (300 mM NaCl for 5 h), drought stress (dehydration for 10 h), and hormones [100  $\mu\text{M}$  abscisic acid (ABA) for 3 h], as well as from the untreated controls. After both conserved and novel miRNAs were identified from all samples, the authors employed RNA gel blot analysis to study miRNA expression change after all four treatments. Their results showed that miR393 was strongly induced by all four tested stress conditions (cold, dehydration, NaCl, and ABA treatments). In contrast, miR389a.1 was inhibited by all of the stress treatments. Interestingly, other miRNAs showed different responses to the various stress treatments. For example, miR319 was induced by cold but not by salinity, dehydration, or ABA (Sunkar and Zhu, 2004). In one of our early studies, we found that 25.8% of ESTs contained one or more miRNAs (Zhang *et al.*, 2005). Although no experiments had confirmed at that time that these miRNAs were only found in stress-induced tissues, the large percentage of ESTs found that contained miRNAs served as an indicator that miRNAs may play some role in plant response to environmental stresses (Zhang *et al.*, 2005). Since these initial studies, the role of miRNAs in plant response to environmental stresses has been attracting attention from many scientists.

miRNA microarrays and deep sequencing technologies have opened the door for investigating which miRNAs are responsive to certain stresses and how much their expression levels change. To date, hundreds of miRNAs have been identified in single plant species. Therefore, it is almost impossible to employ RNA blotting or regular PCR to analyse their expression unless analysis of an individual miRNA is desired. miRNA microarrays, however, can analyse thousands of miRNAs at the same time. Alternatively, deep sequencing not only sequences all of the known miRNAs in a sample but it also sequences all of the small RNAs that are present. Thus, deep sequencing is the most efficient approach to study miRNA expression profiles because it can be used to find new or novel miRNAs that are induced by an individual stress while simultaneously surveying expression levels. Consequently, next-generation high-throughput deep sequencing is currently a popular approach for identifying stress-responsive miRNAs in any plant species, particularly in plant species for which few genome sequence data are available.

### *miRNAs respond to abiotic stress in a genotype-dependent manner*

Over the course of evolution, plants have evolved complicated physiological and genetic mechanisms in order to cope with

and adapt to the harsh environment. It is because of their derivation from a common ancestor that most plants share core gene networks that control plant response to a wide range of environmental factors. However, due to the fact that plants evolved to grow in various environments, plants have also developed numerous regulatory mechanisms for different growth habitats. Even for the same plant species, separate genotypes may show differential gene expression due to deviations in individual plant growth conditions and because of human selection of cultivated crops compared with their wild relatives. Among underlying genetic mechanisms, miRNAs may be one molecule that aids in response to abiotic stress. Like protein-coding genes, many miRNAs also show varied expression from species to species and also from genotype to genotype under certain stress conditions. This difference can be demonstrated by the direction and level of miRNA expression.

miRNA response to abiotic stress in a genotype-dependent manner was evidenced by analysing miRNA expression levels in response to certain stresses among several plant species and cultivars. Several technologies, including deep sequencing, microarrays, quantitative real-time PCR (qRT-PCR) analysis, and even the creation of transgenic plants, showed that miRNA expression profiles varied among plant species. Studies have shown that one miRNA may respond to the same stress differently depending on the plant species, while many miRNAs may respond to the same stress in a similar manner. For example, the expression of miR168 and miR396 was induced in *Arabidopsis* (Liu *et al.*, 2008) and tobacco (Frazier *et al.*, 2011), but was inhibited in rice (Zhou *et al.*, 2010) by drought treatment. On the other hand, drought treatment down-regulated the expression of miR408 in rice (Zhou *et al.*, 2010), peach (Eldem *et al.*, 2012), and cotton (Xie *et al.*, 2015b) but up-regulated miR408 expression in *Arabidopsis* (Liu *et al.*, 2008), *Medicago* (Trindade *et al.*, 2010), and barley (Kantar *et al.*, 2011). Salinity stress induced the overexpression of miR156 in *Arabidopsis* (Liu *et al.*, 2008) but inhibited expression of that same miRNA in maize (Ding *et al.*, 2009). Similarly, plants treated with NaCl highly expressed miR396 in *Arabidopsis* (Liu *et al.*, 2008) and maize (Ding *et al.*, 2009), but not in rice (Zhou *et al.*, 2010). Interestingly, the degree of miRNA response to the same stress varied among plant species although it was either induced or inhibited in all plant species. miRNAs may also respond more in one plant species but less in another. Several studies have identified many species-specific miRNAs that respond to stress treatment. Using deep sequencing, 17 drought-specific miRNAs were identified in switchgrass, of which four were conserved and 13 were switchgrass-specific miRNAs (Xie *et al.*, 2014). In a mechanical stress study, Lu and colleagues (2005) identified 21 miRNA gene families that contained 48 miRNA sequences in *Populus trichocarpa*; among them, only 11 miRNAs were conserved between *P. trichocarpa* and *Arabidopsis*. Interestingly, these conserved miRNAs exhibited species-specific developmental expression patterns, suggesting that even conserved miRNAs may differ in their regulatory roles among plant species. Despite their evolutionary origin (conserved miRNAs or tree-specific

miRNAs), the expression of the majority of poplar miRNAs was altered in a manner consistent with tree-specific corrective growth against tension and compression stresses, which are two constant mechanical loads in trees (Lu *et al.*, 2005). Additionally, Hackenberg and colleagues (2015) identified novel species-specific miRNAs (hvu-miRX33, hvu-miRX34, and hvu-miRX35) in barley that are significantly induced by drought treatment.

The genotype-dependent response of miRNAs to abiotic stresses is not only different among plant species but also varies among cultivars (genotypes) of the same species. It is well known that the genotypes of one plant species may differ in their capacity to respond to abiotic stress. miRNA-mediated gene regulation may contribute to this difference. Using deep sequencing technology, Barrera-Figueroa and colleagues (2011) investigated the impact of drought treatment on two cowpea cultivars (drought-tolerant IT93K503-1 and drought-sensitive CB46). Their results showed that 20 miRNAs were differentially expressed among the two genotypes. Of these miRNAs, nine were predominantly or exclusively expressed in one of the two genotypes but not in the other. Simultaneously, they also identified 11 drought-regulated miRNAs in one genotype but not in the other (Barrera-Figueroa *et al.*, 2011). Using miRNA microarray technology, Yin and colleagues (2012) analysed the miRNA expression profiles of two cotton cultivars with varying resistance to salinity (SN-011 with high tolerance to salinity and LM-6 with sensitivity to salinity). Based on their results, 12 miRNAs were expressed in a genotype-specific pattern. Under salinity treatment, four miRNAs (miR156, miR169, miR535, and miR827) showed significantly high expression in LM-6 whereas the expression of three miRNAs (miR167, miR397, and miR399) was significantly inhibited in this cultivar (Yin *et al.*, 2012). By comparing 12 salinity-tolerant and 12 salinity-susceptible genotypes in rice, Mondal and Ganie (2014) identified 12 miR-SSRs (simple sequence repeats) that were polymorphic. Only miR172b-SSR, however, proved different between the tolerant and susceptible genotype and could therefore serve as a biomarker for distinguishing cultivars with different responses to salinity stress (Mondal and Ganie, 2014). Their results also showed that miRNA genes were less diverse in the tolerant cultivars than in the susceptible cultivars, as evidenced by their calculated polymorphic index content (Mondal and Ganie, 2014). All of these studies suggest that miRNAs may play a role during cultivar-specific response to abiotic stress conditions.

#### *miRNAs respond to abiotic stress in a plant tissue-dependent manner*

To meet the need of a plant, an initiated plant stem cell differentiates into plant tissues/organs with various functions, such as leaves for photosynthesis and roots for taking up water and nutrients from the soil. Thus, it is not surprising that roots are more sensitive to the majority of stresses, particularly those of the soil environment including drought, high salinity, and exposure to pollutants. Similarly, it is not hard to understand that miRNAs in different tissues respond to stress exposure



in different ways. For these reasons, certain stresses in plants tend to induce the expression of miRNAs at higher fold changes in the roots rather than in other plant tissues, such as leaves.

In order to compare root and leaf response to drought stress and to identify genome-wide drought-responsive miRNAs, Eldem and colleagues (2012) constructed four small RNA libraries from both control and treated leaf and root samples and deep sequenced the small RNA populations. The results of this study showed that drought significantly induced the aberrant expression of 262 (104 up-regulated, 158 down-regulated) and 368 miRNAs (221 up-regulated, 147 down-regulated) in leaf and root tissues, respectively (Eldem *et al.*, 2012). Overall, the expression of >100 miRNAs was differentially altered by drought treatment in the roots compared with the leaves (Eldem *et al.*, 2012). Wang and colleagues (2013) also demonstrated that the expression changes of miRNAs were dose and tissue dependent under drought and salinity stress in cotton, in which the tested miRNAs showed altered expression profile patterns in roots compared with the leaves (Wang *et al.*, 2013).

#### *miRNAs respond to abiotic stress in a stress-dependent manner*

There are many types of environmental stresses, including drought, salinity, high temperature, low temperature, UV light, high or low light intensity, hypoxia, heavy metal, nanoparticle, and pollutant exposure, and fertilizer deficiency. Many studies have shown that all of these environmental stresses induce the aberrant expression of miRNAs in a dose- and stress-dependent manner. Interestingly, some miRNAs are commonly responsive to all stresses. Accumulating evidence, however, clearly shows that differential expression of certain miRNAs is dependent on the specific stress condition, even in the same plant species. In *Arabidopsis*, miR169 was inhibited by drought stress (Li *et al.*, 2008) but was found to be induced by salinity treatment (M.Y. Xu *et al.*, 2014). On the other hand, miR398 was induced by UVB light in *Arabidopsis* but was inhibited by salinity, cold, and oxidative stress (Sunkar *et al.*, 2006; Jia *et al.*, 2009). Further experiments have shown that drought-repressed miR169 expression was through an ABA-dependent pathway (Li *et al.*, 2008). The target of miR166, nuclear factor Y (NF-Y) subunit A 5 (NFYA5), was strongly induced by drought stress at the same time that miR169 was inhibited (Li *et al.*, 2008). Both *nfya5* knockout *Arabidopsis* plants and transgenic plants overexpressing miR169 show the same phenotypes, including increased sensitivity to drought stress and enhanced water loss (Li *et al.*, 2008). In contrast, overexpression of *nfya5* in transgenic *Arabidopsis* enhanced plant resistance to drought stress (Li *et al.*, 2008). Interestingly, miR169 was significantly induced by salinity stress in *Arabidopsis*, in which the expression of *nfya5* was inhibited (M.Y. Xu *et al.*, 2014). A similar phenomenon was also observed in other plant species, including soybean (Ni *et al.*, 2013) and rice (Zhao *et al.*, 2007, 2009). Other studies have shown that miR398 was inhibited by both ABA and salinity treatment (Sunkar *et al.*, 2006; Jia

*et al.*, 2009) but was induced by drought treatment (Zhou *et al.*, 2007) in *Arabidopsis*. Analysis of copper superoxide dismutase (CSD) 1 and 2, both targets of miR398, found that both of these genes were induced by salinity treatment (Sunkar *et al.*, 2006; Jia *et al.*, 2009). This suggests that miRNAs may aid plant tolerance to abiotic stresses in a stress-dependent manner.

#### *miRNAs respond to abiotic stress in a miRNA-dependent manner*

While individual miRNAs may respond differently to various stresses in the same plant species, there are many miRNAs whose expression levels can be associated with a particular stress treatment. In *Arabidopsis*, drought treatment induced the expression of many miRNAs, including miR156, miR319, miR393, miR397, and miR408, although the expression fold change differed from miRNA to miRNA (Liu *et al.*, 2008). Some miRNAs, such as miR169, were down-regulated by drought treatment (Liu *et al.*, 2008). Under salinity stress in *Arabidopsis*, the expression of miR156, miR159, miR169, miR319, miR393, and miR397 was significantly induced, although with varying fold changes, but the expression of miR398 was significantly inhibited (Liu *et al.*, 2008).

In summary, studies have shown that environmental abiotic stresses (drought, salinity, high and low temperature, and osmotic stress) induce significant differential expression of miRNAs in a variety of plant species (Fig. 1), including *Arabidopsis* (Liu *et al.*, 2008; Jagadeeswaran *et al.*, 2009; Jia *et al.*, 2009), rice (Zhao *et al.*, 2007), corn (Ding *et al.*, 2009; Kong *et al.*, 2014), poplar (Lu *et al.*, 2005; Jia *et al.*, 2009), and others (Barrera-Figueroa *et al.*, 2011; Budak and Akpinar, 2011; Kulcheski *et al.*, 2011; Li *et al.*, 2011; Qin *et al.*, 2011; Wang *et al.*, 2011). Currently, a number of miRNAs have been reported to be induced by drought and salinity stresses in several different plant species. These miRNAs include miR156, miR159, miR165, miR167, miR168, miR169, miR319, miR393, miR395, miR396, miR398, miR399, and miR402. Almost all of these stress-induced miRNAs are evolutionarily conserved, although evidence supports that miRNAs respond to environmental stresses in a miRNA-, stress-, and genotype-dependent manner. Taken together, this information suggests that miRNAs play a versatile role in plant response to environmental abiotic stresses.

## **miRNA–target gene networks involved in plant response to abiotic stress**

miRNAs do not function directly in plant growth and development or in plant response to environmental stress. Instead, miRNAs participate in plant response to abiotic stresses through regulating key components of complex gene networks. After mature miRNAs are generated, they are loaded into the RNA-induced silencing complex, which contains an argonaute protein, and they then bind to the mRNAs of targeted genes (Winter and Diederichs, 2011; Iwakawa and Tomari, 2013). Based on the complementarity between

miRNA	Maize		Rice			Wheat				Barley		Arabidopsis			Cotton					
	Drought	Salinity	Drought	Salinity	Cold	Heavy m	Drought	Salinity	Cold	UV-B	Drought	Drought	Salinity	Cold	UV-B	Drought	Salinity			
miR156	u	d	D		D	D				U	U	D	U	u	u		u	d	u	
miR157																			d	d
miR159	u		C	D			U	D	C	U	U	u	u			u	d	u	u	
miR160			U	D						U	U					u	d	d		
miR161																		d	d	
miR162	u	u				D												d	d	
miR163																		d	d	
miR164		d	U	D	D		U	D	C	D								d	u	
miR165										D	U				u	u		d	d	
miR166			D		U	D				D	D		C		u	u		d	d	
miR167	u	d	U	D		D				D	D	U						d	d	
miR168	d	u	D		D	D	D		C	C		U	u	u	u	u	u	d	d	
miR169		u	U	U	D				U	D			u	u	u	u	u	d	d	
miR170			D										u	u	u	u	u	u	u	
miR171			C	U	D	D				U	U		U	u	u	u	u	d	d	
miR172			D				U	U	U			D						d	d	
miR319	u		C	D	D				U				u	u	u	u		d	u	
miR390						D												d	d	
miR393			U	U	U			U	U	D		D	u	u	u	u		d	u	
miR394				D	U													u	u	
miR395	d	u	U							D	D	D						d	d	
miR396		d	C	D	U	D						U	u	u	u	u		d	d	
miR397			D				D		D	D		D	u	u	u	u		d		
miR398	u		D						D	D				d	d	u		d	u	
miR399	d								U	U		U						d	d	
miR402			U										u	u	u	u				
miR403																		d	d	
miR408			C	C	U				U	U		U	u		u	u		d	u	
miR417														u				u	u	

**Fig. 1.** A miRNA responds to an environmental abiotic stress in a stress-, species-, and miRNA-dependent manner. Various miRNAs were aberrantly expressed under different abiotic stress treatments in a multiple plant species. Red colour indicates up-regulated (u). Green colour indicates down-regulated (d). Yellow colour indicates that both up-regulation and down-regulation were observed among different tissues or at different developmental stages. The data were based on current literature of *Arabidopsis*, rice, wheat, barley, switchgrass, and cotton.

a miRNA and its targeted mRNA, miRNAs regulate gene expression either by targeting mRNAs for cleavage or by inhibiting protein translation. Usually, when a miRNA perfectly or nearly perfectly aligns with a targeted mRNA, the mRNA will be cleaved at the site on the mRNA corresponding to the site between the 10th and 11th nucleotide of the targeting miRNA. Alternatively, if a miRNA imperfectly binds to the targeted mRNA, the mRNA cannot be translated into protein. In plants, almost all miRNAs perfectly or nearly perfectly bind to their targeted mRNAs; thus, the majority of miRNAs cleave their targeted mRNAs. There have been several reports, however, of plant miRNAs inhibiting protein translation (Rhoades *et al.*, 2002; Bartel, 2004; Zhang *et al.*, 2007). The high degree of complementarity between miRNAs and their targets provides a powerful approach for predicting and validating miRNA target genes in plants. In most cases, a simple BLASTn search can be performed against protein-coding genes using a known miRNA and the results validated using RACE (rapid amplification of cDNA ends)-PCR. Currently, degradome sequencing has been developed and widely used for both miRNA target identification and validation in plants. Table 1 lists the confirmed targets for conserved miRNAs associated with different environmental abiotic stresses.

It is well known that the majority of stress-responsive miRNAs target transcription factors. Transcription factors play an important role during plant response to different environmental stresses. NAM, ATAF, and CUC transcription factors comprise the extensive class of NAC plant-specific

transcription factors. To date, >100 NAC transcription factors have been identified in numerous plant species, including *Arabidopsis* and rice (Nakashima *et al.*, 2012). Recently, several studies have shown that NAC transcription factors play an important role in plant response to various environmental stresses, including drought, salinity, and harsh temperature; correspondingly, overexpression of certain NAC transcription factors significantly enhanced plant tolerance to different abiotic stresses (Mao *et al.*, 2012, 2014; Al Abdallat *et al.*, 2014; Jiang *et al.*, 2014; Pandurangaiah *et al.*, 2014; Q. Xu *et al.*, 2014). More interestingly, the NAC transcription factors are widely targeted by miRNAs. Several studies in *Arabidopsis* and rice have shown that miR164 cleaves NAC mRNAs that modulate plant developmental processes and responses to abiotic stress (Rhoades *et al.*, 2002; Fang *et al.*, 2014). Fang and colleagues (2014) tested six miR164-targeted NAC genes (OMTN1–OMTN6) in rice and found that four of them negatively regulated drought tolerance. SPL transcription factors are master regulators of plant developmental timing and phase change, and have been shown to be post-transcriptionally regulated by miR156 (Wang *et al.*, 2009; Chen *et al.*, 2010). A recent study in *Arabidopsis* found that miR156-mediated down-regulation of SPL increased plant response to environmental stresses, including heat stress and heat stress memory (Stief *et al.*, 2014). Almost all of these miRNAs, as well as their targeted transcription factors, are highly evolutionarily conserved in the plant kingdom, and both the miRNAs and their targeted transcription factors are important for plant development. Therefore, plants

**Table 1.** Common stress-responsive miRNAs and their targets<sup>a</sup>

miRNAs	Targets	References
156/157	SPL	Stief <i>et al.</i> (2014)
159	MYB/TCP	Achard <i>et al.</i> (2004); Reyes and Chua (2007)
160	ARF	Guo <i>et al.</i> (2005)
164	NAC	Rhoades <i>et al.</i> (2002); Fang <i>et al.</i> (2014)
169	NFY	Ni <i>et al.</i> (2013)
173	TAS	Li <i>et al.</i> (2014)
319	TCP	Sunkar and Zhu (2004)
393	TIR	Jones-Rhoades and Bartel (2004); Sunkar and Zhu (2004)
394	F-box	Jones-Rhoades and Bartel (2004)
395	AST	Allen <i>et al.</i> (2005)
395	APS	Jones-Rhoades and Bartel (2004)
396	GRF	Sunkar and Zhu (2004)
397	Laccase	Lu <i>et al.</i> (2013)
398	CSD	Guan <i>et al.</i> (2013); Naya <i>et al.</i> (2014)
402	DML3	Kim <i>et al.</i> (2010b)
	DEMETER-LIKE protein 3)	
828	MYB	Luo <i>et al.</i> (2012)

<sup>a</sup> There have been multiple studies on the targets of individual miRNAs. Here, we only list one or two references for each.

may utilize regulatory mechanisms, such as miRNAs and transcription factors, in order to respond quickly to development cues and stress conditions, and it may be a common mechanism for all plant species to respond to environmental stresses through miRNA-mediated gene targeting of transcription factors.

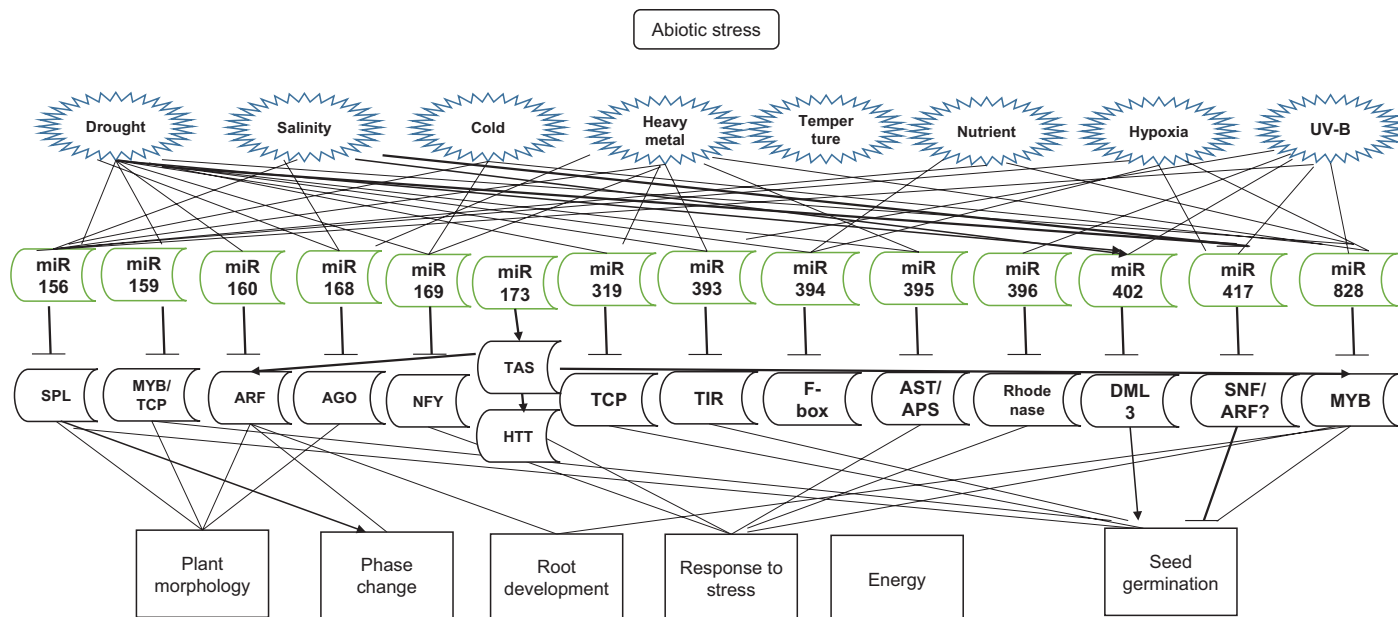
miRNAs respond to different abiotic stresses by targeting stress-responsive genes. Besides transcription factors, there are also lots of other genes associated with and/or responsive to different environmental abiotic stresses, and many of these stress-responsive genes are targeted by an individual miRNA. Laccases are multi-copper-containing glycoproteins that play a role in plant response to abiotic stress by regulating cell wall function during stress conditions (Liang *et al.*, 2006). A recent study showed that laccase genes were targeted by miR397 (Lu *et al.*, 2013). Superoxide dismutase (SOD) is an important enzyme that scavenges ROS, which are usually induced under oxidative stress caused by heavy metal contamination and other abiotic stresses. According to the metal ligands that they bind, there are three types of SODs in plants, iron SOD (Fe-SOD), manganese SOD (Mn-SOD), and copper/zinc SOD (CSD). Loss-of-function mutants of *csd1*, *csd2*, and *ccs* (a copper chaperone of CSD1 and CSD2) exhibited enhanced heat-responsive gene expression in *Arabidopsis* and, overall, the transgenic plants were more heat tolerant (Guan *et al.*, 2013). Additionally, several studies have shown that all three CSD genes are targeted by miR398. Guan and colleagues (2013) generated transgenic *Arabidopsis* plants that expressed miR398-resistant mutants of *csd1*, *csd2*, and *ccs*. The results of this study showed that the miR398-resistant mutants lost the ability to induce aberrant expression of heat-responsive

genes and, therefore, were more sensitive to heat stress (Guan *et al.*, 2013). Other experiments in *Arabidopsis*, as well as in common bean, have also demonstrated that miR398 is overexpressed during heat treatment, which supports other evidence showing that miR398 confers heat tolerance by promoting the cleavage of *csd* mRNAs (Guan *et al.*, 2013; Naya *et al.*, 2014). miR395 functions in abiotic stress response by regulating APS, an important enzyme catalysing the initial activation step of sulphate assimilation (Buchner *et al.*, 2004). Several APS gene family members, including *aps1* and *aps2*, are targeted by miR395 (Jones-Rhoades and Bartel, 2004). In addition to APS, miR395 also targets AST68 (*AtSULTR2;1*, At5g10180), which encodes a sulphate transporter (Allen *et al.*, 2005).

One recent study has shown that *trans*-acting small interfering RNAs (tasiRNA) are generated through miRNA cleavage of TAS mRNAs, and that tasiRNAs are also involved in plant response to abiotic stress (S. Li *et al.*, 2014). Currently, tasiRNAs have been identified in several plant species, including *Arabidopsis* (Peragine *et al.*, 2004; Vazquez *et al.*, 2004), rice (Allen *et al.*, 2005), and cotton (Xie *et al.*, 2014). tasiRNAs are generated from TAS mRNA cleavage by individual miRNAs and, after production, these tasiRNAs target ARF and MYB transcription factors, which are associated with different abiotic stresses. To date, there are at least three miRNAs (miR173, miR828, and miR390) that have been identified that target TAS mRNAs and produce tasiRNAs. miR173-cleaved tasiRNAs target *HEAT-INDUCED TASI TARGET1 (HTT1)* and *HTT2* mRNAs, and have been shown to be involved in thermotolerance in *Arabidopsis* (S. Li *et al.*, 2014), which suggests a new mechanism for miRNAs involved in plant response to abiotic stress.

All this evidence shows that not only are miRNAs involved in plant response to abiotic stress, but that miRNAs function in plant stress response by targeting the transcripts of transcription factors and stress-responsive genes as well tasiRNAs. The entire miRNA gene network forms the basis of the regulatory mechanism (Fig. 2). This network is affected by plant hormones and different signalling pathways. miRNAs are also involved in the adaptation of roots to various environmental abiotic stresses by targeting genes within this network. Environmental conditions of the soil affect both primary root and lateral root development, and both of these are also partially affected by the miRNA-mediated gene network response. One of most important factors influencing root development is the supply of nutrients. Since nutrients are most probably located in the upper part of the soil, a deficiency in fertilizer usually causes an increase in lateral root development. This is evidenced by an increase in lateral root length and density, and also by a decrease in primary root extension. Using cell-sorting experiments, Gifford and colleagues (2008) found that *Arabidopsis* roots responded to nitrogen deficiency in a cell-specific manner. Similarly, after adding nitrate to nitrogen-depleted *Arabidopsis* plants, the expression of miR167 was decreased, which resulted in an increase of its targeted gene, ARF8, particularly in the pericycle cells (Gifford *et al.*, 2008). These plants also displayed increased lateral root initiation, emergence, and development (Gifford *et al.*, 2008). Drought





**Fig. 2.** The miRNA–target gene network is involved in plant response to environmental abiotic stresses. Different stresses induced and/or inhibited the expression of individual miRNAs that target transcription factors and/or stress-related genes. This network further regulates plant development as well as response to abiotic stress. Plant hormones are also involved in this process through directly/indirectly regulating the expression of miRNAs and their targets. (This figure is available in colour at *JXB* online.)

and salinity stress are also known to affect root development, partially by way of miRNA-mediated gene regulation. There are many miRNAs that respond to different environmental stresses, and many of these stress-responsive miRNAs also modulate root development by targeting root development-related genes and transcription factors, such as NACs, ARFs, and HD-ZIPs.

### Phytohormones and their corresponding signalling pathways regulate miRNA–target gene networks and contribute to plant response to abiotic stress

Plant hormones play a central role in plant adaptation to abiotic stress by regulating plant growth, development, nutrient allocation, and source/sink transitions (Peleg and Blumwald, 2011). Almost all plant hormones, including ABA, auxin, cytokinins, gibberellic acid (GA), ethylene, brassinosteroids, and jasmonic acid (JA), along with their corresponding signalling pathways, are involved in plant response to different environmental abiotic and biotic stresses. Cross-talk among different plant hormones, stress-related genes, and signalling pathways, as well as recently discovered miRNAs that are involved, results in synergistic or antagonistic interactions during plant response to abiotic stress (Peleg and Blumwald, 2011).

ABA is a stress-responsive plant hormone. Under stress conditions, ABA usually accumulates within the plant, which enhances the production of ROS. Together, ABA and ROS accumulation function as a stress signal that then induces the expression of mitogen-activated protein kinases (MAPKs) and antioxidant genes, ultimately enhancing plant

tolerance to the stress condition. In maize seedlings, drought treatment inhibited the expression of miR168 and miR528, which resulted in overexpression of their target MAPK and peroxidase genes, and consequently overlapped with the ABA-involved signalling pathway (Wei *et al.*, 2009). Drought conditions also induced stomatal movement and antioxidant defence in maize, which enhanced maize tolerance to drought stress (Wei *et al.*, 2009).

miRNAs function in stress-related auxin signalling by modulating the expression of auxin response factor (ARF) transcription factors, which are involved in root development and stress response. At least five miRNAs (miR160, miR164, miR167, miR390, and miR393) have been shown to be involved in this network. For example, miR160 targets ARF10, ARF16, and ARF17, while miR167 interacts with ARF6 and ARF8, and miR390 targets ARF4. On the other hand, miR164 and miR393 are indirectly involved in the regulation of ARFs. All of these miRNAs and ARFs participate in root development and response to stress treatment (Meng *et al.*, 2010). One study has suggested that auxin-induced miR164 functions to provide a homeostatic balance within the auxin signalling pathway by inhibiting *NAC1*, a transcription factor, and down-regulating auxin signalling (Guo *et al.*, 2005).

The MAPK signalling pathway is also part of the miRNA–target gene network. MAPK signalling cascades are one of the most conserved pathways in plants and are known to regulate plant development as well as response to abiotic and biotic stresses (Raghuram *et al.*, 2014). A recent study has shown that 98 out of 99 rice MAPK genes are potentially targeted by certain miRNAs, and the expression levels of these MAPKs were inversely correlated with the expression levels of their predicted miRNAs (Raghuram *et al.*, 2014).

## miRNAs, a new target for improving plant tolerance to abiotic stress

miRNAs play a significant role in plant growth and development and, recently, these molecules have emerged as important players in plant response to various environmental abiotic stresses. Further analysis has shown that miRNAs are located at the centre of complicated gene regulatory networks. Thus, miRNAs are becoming a novel target for plant improvement, including enhanced tolerance to different stresses (Zhang and Wang, 2015). To date, several miRNAs have been overexpressed in multiple plant species. Depending on their target genes, the miRNA-overexpressing transgenic plants exhibited either higher tolerance or sensitivity to different environmental abiotic stresses as compared with their wild type (Table 2). Several of these transgenic plants showed promise for using miRNA-based biotechnology for enhancing plant tolerance to harsh environments.

miR156 was the first miRNA identified in plants and it has been shown to play a critical role in plant development and phase change. Recent studies demonstrated that miR156 was aberrantly expressed during plant exposure to various environmental stresses. Transgenic approaches have been used for validating miR156 function. miR156-overexpressing plants revealed that miR156 was required for plant heat stress memory, and these plants exhibited enhanced tolerance to heat stress (Stief *et al.*, 2014). miR156 has also been used to improve plant biomass. Transgenic switchgrass that overexpressed the appropriate miR156 produced 58–101% more plant biomass in comparison with their control plants (Fu *et al.*, 2012).

miR159 plays an important role in plant development. miR159 responded to various environmental stresses, and one study found that transgenic rice plants overexpressing miR159 were more sensitive to heat stress in comparison with the wild-type controls, suggesting that down-regulation of miR159 may contribute to heat stress tolerance (Wang *et al.*, 2012).

miR169 is one of the largest miRNA families that is conserved in all plant species. miR169 is a significant contributor to proper plant development and also in plant response to environmental stress. Constitutive overexpression of miR169 in transgenic tomato significantly enhanced plant tolerance to drought stress after 7 d of drought treatment (Zhang *et al.*, 2011). During drought treatment, non-transgenic wild-type tomato plants showed obvious dehydration symptoms, including wilting and turgor loss; however, the transgenic plants that overexpressed miR169 grew very well (Zhang *et al.*, 2011). This study showed that transgenic tomato plants reduced their stomatal aperture index by 35–49% and their stomatal conductance by 33–45% (Zhang *et al.*, 2011). Additionally, the transpiration rate of transgenic plants was reduced by 38–55% when compared with wild type non-transgenic tomato plants (Zhang *et al.*, 2011). Thus, transgenic plants that overexpressed miR169 had reduced water loss through the leaves and required less water from the soil (Zhang *et al.*, 2011). Overexpression of miR169 also caused transgenic *Arabidopsis* plants to be hypersensitive to nitrogen

**Table 2.** A list of studies that overexpressed miRNAs in order to alter plant tolerance to environmental stresses

Targeted miRNAs	Transgenic plants	Targeted stress	References
156	<i>Arabidopsis</i>	Tolerance to heat stress	Stief <i>et al.</i> (2014)
156	Switchgrass	Increase of biomass	Fu <i>et al.</i> (2012)
159	Rice	Sensitive to heat stress	Wang <i>et al.</i> (2012)
169	Tomato	Enhancing plant tolerance to drought	Zhang <i>et al.</i> (2011)
169	<i>Arabidopsis</i>	Sensitivity to nitrogen deficiency	Zhao <i>et al.</i> (2011)
173	<i>Arabidopsis</i>	Thermotolerance	Li <i>et al.</i> (2014)
319	Bentgrass	Tolerance to salinity and drought	Zhou <i>et al.</i> (2013)
319	Rice	Tolerance to chilling temperature	Yang <i>et al.</i> (2013)
393	<i>Arabidopsis</i>	More sensitive to salinity and alkalinity	Gao <i>et al.</i> (2011)
393	Rice	More sensitive to salinity and alkalinity	Gao <i>et al.</i> (2011)
394	<i>Arabidopsis</i>	Tolerance to drought	Ni <i>et al.</i> (2012)
395	<i>Arabidopsis</i>	Drought and salinity stress	Kim <i>et al.</i> (2010a)
395	Rapeseed	Enhanced tolerance to oxidative stress and heavy metal stress	Zhang <i>et al.</i> (2013)
396	<i>Arabidopsis</i>	More sensitive to salinity and alkalinity	Gao <i>et al.</i> (2010)
396	Rice	More sensitive to salinity and alkalinity	Gao <i>et al.</i> (2010)
402	<i>Arabidopsis</i>	More tolerance to salinity, drought, and cold stress	Kim <i>et al.</i> (2010b)
417	<i>Arabidopsis</i>	More sensitive to salinity and ABA	Jung and Kang (2007)
828	Sweet potato	Oxidative stress	Lin <i>et al.</i> (2012)

starvation, as evidenced by the yellowing of transgenic leaves under all tested nitrogen starvation conditions (Zhao *et al.*, 2011). This suggests that miR169 also targets genes that function in nitrogen assimilation. Therefore, miR169 is a promising target for improving plant tolerance to drought stress and nitrogen deficiency.

miR319 has been identified to be associated with multiple abiotic stresses, and several studies have shown that miR319 is usually up-regulated during multiple stress conditions (Sunkar and Zhu, 2004; Zhou *et al.*, 2010). A transgenic study found that constitutive expression of miR319 in creeping bentgrass significantly enhanced plant tolerance to salinity and drought stress, and also altered plant development (Zhou *et al.*, 2013). This enhanced abiotic stress tolerance could be attributed to the regulation mechanism of miR319, since it is known to cleave the mRNAs of TCP transcription factors (Pieczynski *et al.*, 2013). The transgenic plants that



overexpressed miR319 exhibited increased water retention and cell membrane integrity when compared with their non-transgenic controls (Pieczyński *et al.*, 2013). Under salinity stress conditions, the transgenic plants also accumulated less Na<sup>+</sup> compared with the wild-type plants (Pieczyński *et al.*, 2013). Overexpression of miR319 in transgenic rice was found to enhance rice tolerance to cold stress significantly (Yang *et al.*, 2013). In this study, 7-day-old rice seedlings were acclimated to cold conditions by placing transgenic and wild-type seedlings at 12 °C for 2 d followed by 4 °C for 4 d (Yang *et al.*, 2013). At the end of the experiment, transgenic rice that overexpressed miR319 had a higher survival rate (~50%) compared with the wild-type controls (13% of survival rate) (Yang *et al.*, 2013). Taken together, the results of these studies suggest that overexpression of miR319 can be used to enhance plant tolerance to multiple environmental stresses.

Another conserved miRNA family that exists in both monocot and dicot plants and whose expression is altered by many environmental stresses is miR393. Thus, miR393 has become another target for improving plant tolerance to different stresses. Gao and colleagues (2011) transformed miR393 into rice and *Arabidopsis* under control of the *Cauliflower mosaic virus* (CaMV) 35S promoter. Transgenic and control plants were subjected to salinity (150 mM NaCl) and alkalinity (75 mM NaHCO<sub>3</sub>) treatments for 15 d (Gao *et al.*, 2011). The results of this study showed that the T<sub>3</sub> generation seedlings of miR393-overexpressing transgenic *Arabidopsis* were more sensitive to salinity and alkalinity treatment in comparison with wild-type plants. Likewise, the transgenic rice plants exhibited a similar phenotype (Gao *et al.*, 2011). For both *Arabidopsis* and rice, overexpression of miR393 significantly inhibited seedling growth and root development (Gao *et al.*, 2011).

miR394 is also evolutionarily conserved, and recent studies in *Arabidopsis* have demonstrated that the expression of miR394 is significantly induced by high salinity treatment and is also altered by iron and sulphate deficiency (Liu *et al.*, 2008; Kong and Yang, 2010). Under drought conditions, transgenic *Arabidopsis* plants that overexpressed miR394 restricted water loss during leaf transpiration, which ultimately increased tolerance to drought stress (Ni *et al.*, 2012).

miR395 has been shown to exhibit aberrant expression under different stress conditions. Transgenic approaches to elucidate miR395 function have found that overexpression of miR395 affects plant tolerance to salinity and drought stress, as evidenced by a decrease in seed germination and seedling growth (Kim *et al.*, 2010a). However, members of the miR395 family may regulate different targets during these processes due to a single nucleotide difference between their mature miRNA sequences. Hence, miR395 can also function in plant tolerance to heavy metals. Transgenic rapeseed that overexpressed miR395 showed a higher tolerance to cadmium stress (L.W. Zhang *et al.*, 2013). This was evidenced by a lower degree of cadmium-induced oxidative stress in the transgenic plants compared with their wild-type controls (L.W. Zhang *et al.*, 2013). The higher tolerance to cadmium stress could

be attributed to the fact that the transgenic plants contained higher chlorophyll, glutathione, and non-protein thiols (L.W. Zhang *et al.*, 2013).

miR396 is a known stress-responsive miRNA. During salinity and alkalinity stress treatments, transgenic *Arabidopsis* and rice plants that constitutively overexpressed miR396 had significantly stunted root growth as well as decreased plant growth and development (Gao *et al.*, 2010). Interestingly, no phenotypic differences were observed between transgenic miR396-overexpressing *Arabidopsis* seedlings and wild-type plants under drought stress conditions. This suggests that miR396 is a negative regulator of plant response to salinity and alkalinity stresses.

miR402 has been shown to be up-regulated in *Arabidopsis* by salinity, dehydration, and cold stresses (Sunkar and Zhu, 2004; Kim *et al.*, 2010b). Overexpression of miR402 promoted seed germination in *Arabidopsis* under all three stress conditions; however, transgenic plants that overexpressed miR402 only exhibited enhanced plant growth under salinity stress but not under dehydration or cold stress conditions (Kim *et al.*, 2010b).

Additionally, salt and dehydration stresses altered the expression of miR417 (Jung and Kang, 2007). Constitutive overexpression of miR417 negatively impacted seed germination and survival of *Arabidopsis* seedlings under high salinity stress conditions and in the presence of ABA (Jung and Kang, 2007).

miR828 is a wound-induced miRNA. Interestingly, miR828 expression was induced by wounding but not by ethylene, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), methyl jasmonate, or nitric oxide (NO) (Lin *et al.*, 2012). Transgenic sweet potato overexpressing pre-miR828, however, exhibited an increase in lignin biosynthesis and the production of H<sub>2</sub>O<sub>2</sub>, two components that are generated to promote plant defence mechanisms (Lin *et al.*, 2012).

The majority of transgenic technology studies have focused on the effects of stress conditions on seed germination and seedling growth. Since desirable crops may be allocated to grow on compromised lands, seed germination and seedling establishment will be critical for the survival, health, and overall yield of the crop. Therefore, all of the aforementioned transgenic studies, in which individual miRNAs were overexpressed, provide strong evidence for the use of a novel miRNA-based biotechnology for improving crop tolerance to various environmental stresses (Zhang and Wang, 2015).

## Conclusions and perspectives

Over the course of time, numerous scientists have tried to elucidate the mechanisms underlying plant response to abiotic stress. In order to support the world's growing human population, future endeavours will be needed that will focus on breeding new crop cultivars with high tolerance to different environmental stress conditions. Although great progress has been made over the past 20 years, including the identification of both protein-coding genes and small RNAs responsive to

abiotic stress, all of these studies are still in their infancy and, therefore, more time is needed before miRNAs become a real target for improving crop tolerance.

#### *miRNA-related research needs to focus on functional analysis instead of miRNA identification*

In the past decade, particularly with the development of computational programs and the advancement of deep sequencing technologies, the majority of plant miRNA-related research has focused on the identification of miRNAs from different plant species; however, there are only a few studies that have aimed to elucidate the functions of these miRNAs. Therefore, future studies in this field should switch from identifying miRNAs that are responsive to abiotic stresses to validating the roles of individual miRNAs in plant tolerance to stress conditions. In order to achieve this goal, new technologies must be developed that are able to screen and test multiple genes, including miRNAs, at the same time. This is especially important for miRNAs because there are many miRNAs that have been identified to be responsive to abiotic stress. One new approach may include virus-induced gene silencing (VIGS). Recently, one study successfully overexpressed miR156 in cotton using *Cotton leaf crumple virus* (CLCrV)-induced gene silencing, which resulted in abnormal leaf development in the transgenic plants (Gu *et al.*, 2014). This study also used VIGS technology to transform a miR156 small tandem target mimic (STTM) into cotton in order to knock down a target of miR156 (Gu *et al.*, 2014). Thus, VIGS can be an efficient approach for studying the function of individual miRNAs.

#### *Using both miRNAs and artificial miRNAs (amiRNAs) to develop transgenic plants with high tolerance to abiotic stress*

More and more studies are showing that abiotic stresses induce the aberrant expression of miRNAs and that miRNAs sit at the hub of gene networks that regulate plant response to abiotic stress. This leads to the promise of utilizing miRNAs as a new target for genetically improving agriculturally important traits, including plant response to environmental stress. However, only a few studies have been performed that transformed targeted miRNAs into crops with the goal of improving crop stress tolerance. Additionally, only a few reports have focused on validating miRNA function. One reason why this type of research may be hindered is because we have limited knowledge of the miRNA-regulated gene network that is involved in plant response to abiotic stress. The use of artificial miRNAs (amiRNAs), which can target stress-responsive mRNAs, may solve this problem. In a recent study, amiRNAs were designed to knock down nuclear cap-binding protein 80 [CBP80; also known as Abscisic Acid Hypersensitive 1 (ABH1)] in potato (Pieczynski *et al.* 2013). The results of this study showed that transgenic potato plants exhibited a higher tolerance to drought stress, particularly due to an increase in leaf stomata and trichome density, ABA-hypersensitive stomatal closing, and compact cuticle structures containing a lower number of microchannels (Pieczynski *et al.*, 2013).

#### *Is miRNA response to abiotic stress a common mechanism or a genotype/stress-dependent mechanism?*

It is obvious that different kinds of environmental stress induce the aberrant expression of certain miRNAs in all plant species. Although many studies have been performed in this field, particularly on individual plant species, no study has systemically investigated the common and non-common miRNA-regulated mechanisms that occur during various stresses across a variety of plant species. Therefore, miRNA-mediated reactions may or may not be genotype/stress dependent. To test this, a large-scale study should be performed that compares miRNA expression profiles among several plant species during environmental stress conditions. Ultimately, it will be better to compare major agricultural crops (e.g. maize, soybean, and cotton) with model plant species (e.g. *Arabidopsis*) so that knowledge gathered from the model plants can be directly translated into functional studies for improving crop tolerance to environmental stress.

#### *Is miRNA modification involved in plant response to abiotic stress?*

During miRNA biogenesis, miRNAs may go through several modifications, including truncation, addition, and nucleotide substitution. Several recent reports have shown that miRNA modification widely exists in plants (Xie *et al.*, 2015c; Zhai *et al.*, 2013; J. Zhang *et al.*, 2013), including agriculturally important crops (Xie *et al.*, 2015c). Although one study has proposed that miRNA modification may play an important role in miRNA regulation of plant growth and development, no experimental study has been performed to elucidate the function of individually modified plant miRNAs. Functional analysis of miRNA modifications may provide new insights into plant miRNA biogenesis and ultimately may aid in generating plants with enhanced tolerance to abiotic stress.

## Acknowledgements

Mr Jun Ma and Ms Runrun Sun helped to collect some data on miRNA expression in different plant species under different abiotic stresses. We appreciate Ms Taylor Frazier of Virginia Polytechnic Institute and State University for critically proofreading this manuscript. The related project has been partially supported by the USDA NIFA, Cotton Incorporated, and the North Carolina Biotechnology Center.

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