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Role of microRNAs in plant drought tolerance

Jannatul Ferdous, Syed S. Hussain and Bu-Jun Shi*

Australian Centre for Plant Functional Genomics, The University of Adelaide, Urrbrae, SA, Australia

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**Correspondence* (Tel 61 8 83137168;
fax 61 8 83137102;
email bujun.shi@adelaide.edu.au)

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Summary

Drought is a normal and recurring climate feature in most parts of the world and plays a major role in limiting crop productivity. However, plants have their own defence systems to cope with adverse climatic conditions. One of these defence mechanisms is the reprogramming of gene expression by microRNAs (miRNAs). miRNAs are small noncoding RNAs of approximately 22 nucleotides length, which have emerged as important regulators of genes at post-transcriptional levels in a range of organisms. Some miRNAs are functionally conserved across plant species and are regulated by drought stress. These properties suggest that miRNA-based genetic modifications have the potential to enhance drought tolerance in cereal crops. This review summarizes the current understanding of the regulatory mechanisms of plant miRNAs, involvement of plant miRNAs in drought stress responses in barley (*Hordeum vulgare* L.), wheat (*Triticum* spp.) and other plant species, and the involvement of miRNAs in plant-adaptive mechanisms under drought stress. Potential strategies and directions for future miRNA research and the utilization of miRNAs in the improvement of cereal crops for drought tolerance are also discussed.

Introduction

Drought is one of the most common environmental stresses affecting growth, development and yield of plants (Ceccarelli and Grando, 1997). Understanding plant tolerance to drought is important for the improvement of crop productivity (Lawlor, 2013). During evolution, plants have developed different defence strategies against drought. One of them is to escape the drought by timing the most sensitive stages of development (e.g. reproductive stage) to occur when the stress is less severe. Another strategy is drought avoidance, involving maintenance of high tissue water potential. The third strategy combines enhanced water acquisition using a deep root system with minimization of water loss by restraining transpiration. Mechanisms of drought tolerance include maintenance of turgor through osmotic adjustment, increased cell elasticity and decreased cell size as well as desiccation tolerance via protoplasmic tolerance. In molecular terms, many genes have been implicated in drought tolerance (Shinozaki and Yamaguchi-Shinozaki, 2007). However, transgenic plants overexpressing some drought-responsive genes did not exhibit significant improvements or had no improvement at all for drought tolerance (Bartels and Sunkar, 2005). This may reflect the fact that the plant drought stress responses, tolerance mechanisms and genetic control of tolerance are complex.

Expression of microRNAs (miRNAs) has been found to be altered in plants during drought stress. This finding helps shed light on drought response mechanisms which can potentially be targeted in development of new drought tolerant crops (Chen *et al.*, 2012; Kantar *et al.*, 2010; Niu *et al.*, 2006; Zhao *et al.*, 2007). The focus of this review is to provide an update on microRNAs and their involvement in responses to stresses, particularly in cereal crop species against drought. Firstly, we outline the knowledge on biogenesis and functions of plant miRNAs. Another section addresses the behaviour and roles of miRNAs under drought stress in barley and wheat. Then, the work regarding the involvement of miRNAs in potential drought-adaptive mechanisms of plants is discussed. Finally, we discuss the scope for utilizing miRNAs for improving drought tolerance of crop plants, especially barley and wheat.

MiRNAs: discovery, biogenesis and mechanisms

miRNA were first discovered in the nematode *Caenorhabditis elegans* in 1993 at which time they were considered as small temporal RNAs (stRNAs; Lee *et al.*, 1993). In 2001, miRNAs were formally named and recognized as a distinct class of RNAs with regulatory functions (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001). Plant miRNAs were identified 10 years after animal miRNAs (Reinhart *et al.*, 2002). Now, 7385 mature miRNAs and 6150 precursor miRNAs (pre-miRNAs) have been identified in 72 plant species (miRBase, 20 June 2013; Griffiths-Jones *et al.*, 2008). miRNAs are single-stranded noncoding RNAs sized usually between 20 and 24 nucleotides (nt) that serve as gene regulators in a wide range of organisms (Lee *et al.*, 1993; Reinhart *et al.*, 2002; Shabalina and Koonin, 2008). They affect many biological processes including development of organs such as roots, stems, leaves and flower parts (Bartel, 2004; Bian *et al.*, 2012; Chen, 2004; Chen *et al.*, 2011; Kim *et al.*, 2005; Liu and Chen, 2009; Maizel and Jouannet, 2012; Ronemus and Martienssen, 2005; Vaucheret *et al.*, 2004; Wang *et al.*, 2005, 2008). A growing body of evidence suggests that miRNAs play key roles in plant responses to biotic and abiotic stresses. miRNAs mediate the responses by modulating the amount of themselves, the amount of mRNA targets or the activity/mode of action of miRNA–protein complexes. In turn, these changes modify the

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timing, location and amount of proteins expressed from other genes upon exposure to the stress. Much of the gene regulation by miRNAs in response to plant biotic and abiotic stresses occurs at the post-transcriptional level (Ding *et al.*, 2013; Feng *et al.*, 2013; Floris *et al.*, 2009; Gupta *et al.*, 2012; Jian *et al.*, 2010; Liu *et al.*, 2008, 2012; Ozhuner *et al.*, 2013; Sunkar *et al.*, 2006; Wang *et al.*, 2014; Xie *et al.*, 2012; Yang *et al.*, 2012; Zhang *et al.*, 2009, 2011). In this section, we briefly describe how miRNAs are generated and functional in organisms.

miRNAs are transcribed from genes, but the transcripts are not translated into proteins. The primary transcript (pri-miRNAs) base pairs with itself to create a structure containing stem-loop and double-stranded RNA sections, and which is then processed by an RNase III enzyme [called Dicer-like1 (DCL1) in plants and Drosha in animals] into stem-loop structures of approximately 70 nt (pre-miR NAs). The release of a miRNA/miRNA* duplex from a pre-miRNA is carried out in the nucleus by DCL1 in both plants and animals. 'miRNA' refers to the strand that will become the miRNA, and 'miRNA*' refers to the strand that is complementary to the miRNA. Each strand is methylated to avoid degradation before being transported to the cytoplasm where the miRNA strand of the miRNA/miRNA* duplex is incorporated into a protein complex known as RISC (RNA-induced silencing complex), which is a multiprotein complex that incorporates one strand of miRNA or a small interfering RNA (siRNA). For a detailed description of miRNA biogenesis, readers are referred to the studies by Bao *et al.* (2004), Baumberger and Baulcombe (2005), Khraiwesh *et al.* (2010), Lee *et al.* (2004), Park *et al.* (2005), Voinnet (2009), and Wu *et al.* (2010).

Plant miRNAs are derived by processing of their RNA precursors. Such precursors are occasionally transcribed from an intron or exon of a protein coding region, but most precursors are transcribed from the intergenic regions of genomes (Chen, 2004; Jones-Rhoades *et al.*, 2006; Kim, 2005; Reinhart *et al.*, 2002). miRNA biogenesis involves multiple steps to form mature miRNAs from miRNA genes (Chen, 2009; Jones-Rhoades *et al.*, 2006; Kim, 2005; Park *et al.*, 2005; Voinnet, 2009). Mirtrons, a type of miRNAs, originating from the introns can bypass the microprocessor complex (a multisubunit complex comprising the RNAse III enzyme) and directly enter as pre-miRNA into the miRNA maturation pathway (Zhu *et al.*, 2008). A few miRNAs can be generated independently of the splicing pathway, but details of their maturation are obscure (Johanson *et al.*, 2013). Both strands of a miRNA duplex can be incorporated into the Argonaute (AGO)-containing RISC complex used for silencing the target (Okamura *et al.*, 2008). From this fact, defining which strand is miRNA and which strand is miRNA* is difficult. Perhaps for this reason, currently, the miRNA and miRNA* terms are widely replaced by the '3p' and '5p' suffixes according to their positions in the precursor miRNAs (pre-miRNAs). Surprisingly, loop-derived miRNAs were recently identified and shown to be functional (Hackenberg *et al.*, 2013; Okamura *et al.*, 2013; Winter *et al.*, 2013). However, how these loop-derived miRNAs are generated has not yet been elucidated. Once a miRNA is incorporated into the RISC, it would guide AGO by base pairing with mRNA to cleave the target (Baumberger and Baulcombe, 2005; Jones-Rhoades *et al.*, 2006) or inhibit translation of the target (Arteaga-Vazquez *et al.*, 2006; Aukerman and Sakai, 2003; Brodersen and Voinnet, 2009; Brodersen *et al.*, 2008; Chen, 2004; Gandikota *et al.*, 2007; Gu and Kay, 2010; Vazquez *et al.*, 2010). While miRNA mediated post-transcriptional gene regulation is common, in human cells miRNAs also regulate genes at the transcriptional level (Kim *et al.*, 2008; Place *et al.*, 2008). The mechanism of action is unclear, but it is likely to be via miRNA-directed DNA methylation, which occurs at cytosine in all sequence contexts (Axtell, 2013; Chellappan *et al.*, 2010; Khraiwesh *et al.*, 2010; Vazquez *et al.*, 2008; Wu *et al.*, 2009, 2010; Zhu *et al.*, 2008). This way of regulation is very similar to siRNA-directed DNA methylation (Chan *et al.*, 2005; Matzke *et al.*, 2009). siRNA is a class of double-stranded small RNAs of 21–24 base pairs in length, which plays important roles in the RNA interference (RNAi) pathway. In contrast to siRNAs, miRNA-directed DNA methylation is affected by multiple factors such as the Dicer member (Khraiwesh *et al.*, 2010), miRNA size (Wu *et al.*, 2010), AGO member (Axtell, 2013; Chellappan *et al.*, 2010; Khraiwesh *et al.*, 2010; Vazquez *et al.*, 2008; Wu *et al.*, 2010) and stability of the duplex miRNA (Khraiwesh *et al.*, 2010). In plants, miRNAs mainly function at the post-transcriptional gene silencing (PTGS) level and guide the AGO protein to cleave the target mRNA between positions 10 and 11 (relative to the 5′ end of the miRNA). Although the exact way of translational inhibition is still obscure, it is assumed that, during translation, miRNAs do not result in mRNA cleavage, but arrest translation by blocking read-through of the ribosome (Wang *et al.*, 2008). A recent study showed that miRNA-directed translation inhibition occurs at the endoplasmic reticulum (ER) and requires ALTERED MERISTEM PROGRAM1 (AMP1) (Li *et al.*, 2013). Given that homologues of AMP1 are present in animal genomes, it is possible that the connection between the ER and translation inhibition by miRNAs is conserved across plants and animals (Li *et al.*, 2013). Taken together, it is suggested that miRNAs may regulate the expression of their target genes via a combination of the aforementioned mechanisms (Eulalio *et al.*, 2008). Large amounts of data have indicated that miRNA regulatory activity has effects on growth and development as well as on responses to environmental stresses (Berger *et al.*, 2009; Khraiwesh *et al.*, 2012; Llave, 2004; Meng *et al.*, 2009; Reyes and Chua, 2007; Rodriguez *et al.*, 2010; Schommer *et al.*, 2008; Sunkar and Zhu, 2004). As the biogenesis and functional mechanism of miRNAs have already been reviewed extensively, we will not discuss those aspects further.

miRNA responses to drought stress

Drought stress has been revealed to alter expression of many genes/metabolites, including dehydrins, vacuolar acid invertase, glutathione S-transferase (GST), abscisic acid (ABA)-inducible genes [LEA (late embryo abundant), RAB (responsive to abscisic acid), COR (cold regulated), Rubisco (5-bisphosphate carboxylase-oxygenase)], helicase, proline and carbohydrates (Nezhadahmadi *et al.*, 2013; references therein). miRNAs as gene regulators are expected to participate in the regulation of these drought-responsive genes. Studies have shown that the expression of miRNAs is themselves altered in response to drought stress. Drought-responsive miRNAs have been reported in many plant species such as *Arabidopsis* (Sunkar and Zhu, 2004), rice (Zhou *et al.*, 2010), cowpea (Barrera-Figueroa *et al.*, 2011), tobacco (Frazier *et al.*, 2011), soya bean (Kulcheski *et al.*, 2011), *Phaseolus vulgaris* (Arenas-Huertero *et al.*, 2009) and so on and have been summarized in Table 1. In *Arabidopsis*, miR156, miR159, miR167, miR168, miR171, miR172, miR319, miR393, miR394a, miR395c, miR395e, miR396 and miR397 are up-regulated, while miR161, miR168a, miR168b, miR169, miR171a and miR319c are down-regulated, under drought stress (Liu *et al.*,

Table 1 Drought-responsive miRNAs in different plant species

miRNA	Target name and functions*	Species†	Source
miR156	SBP family of transcription factors—promote phase transitions, flowering time	*Ath*↑, *Tdi*↑, *Hvu*↑, Rice↓ *Peu*↑, *Ppe* (slightly)↑, *Pto*↓	Eldem *et al.* (2012), Kantar *et al.* (2011), Liu *et al.* (2008), Ren *et al.* (2012), Wu and Poethig (2006) and Zhou *et al.* (2010)
miR157	SBP family of transcription factors	*Ppe*↑↓	Eldem *et al.* (2012)
miR159	MYB and TCP transcription factors—ABA response, Nacl stress response, floral asymmetry and leaf development	*Ath*↑ Rice↓ *Ppe*↓	Arenas-Huertero *et al.* (2009), Eldem *et al.* (2012), Jones-Rhoades and Bartel (2004), Liu *et al.* (2008), Reyes and Chua (2007) and Zhou *et al.* (2010)
miR160	ARF 10, ARF 16 and ARF 17—seed germination and postgermination stages	*Ppe*↑, *Pto*↑, *Ptc*↓	Eldem *et al.* (2012), Jones-Rhoades and Bartel (2004), Liu *et al.* (2007), Ren *et al.*, (2012) and Shuai *et al.* (2013),
miR162	DCL1—miRNA biogenesis	*Pto*↑	Ren *et al.* (2012) and Xie *et al.* (2003)
miR164	NAC domain TF—lateral root development	*Mtr*↓, *Ptc*↓, *Bdi*↓	Shuai *et al.* (2013) and Wang *et al.* (2011)
miR165	HD-ZIPIII transcription factor—axillary meristem initiation, leaf and vascular development	*Ppe*↓	Eldem *et al.* (2012)
miR166	HD-ZIPIII transcription factor—axillary meristem initiation, leaf and vascular development	*Tdi*↓, *Gma*↑	Kantar *et al.* (2011), Li *et al.* (2011a,b), Sun (2012) and Williams *et al.* (2005)
miR167	ARF6 and ARF8—gynoecium and stamen development	*Ath*↑, *Ppe*↓, *Pto*↑	Eldem *et al.* (2012), Liu *et al.* (2008), Ren *et al.* (2012) and Wu and Poethig (2006)
miR168	ARGONAUTE1, MAPK—miRNA biogenesis and mRNA degradation, plant development	*Ath*↑ Rice↓ *Z. mays*↓	Liu *et al.* (2008), Wei *et al.* (2009) and Zhou *et al.* (2010)
miR169	NF-YA transcription factor subunit A-3, NF-YA transcription factor subunit A-10, SlMRP1—Plant development and Flowering timing, response to different abiotic stresses	*Ath*↓, Tomato↑, Rice↑, *Mtr*↓, *Ppe*↓, *Gma*↑, *Pto*↓, *Peu*↑	Eldem *et al.* (2012), Li *et al.* (2008), Li *et al.* (2011a,b), Qin *et al.* (2011), Ren *et al.* (2012), Trindade *et al.* (2010), Wang *et al.* (2011), Zhang *et al.* (2011), Zhao *et al.* (2007) and Zhou *et al.* (2010)
miR170	SCL transcription factor—radial patterning in roots, floral development and shoot branching	*Ath*↓, Rice↓	Sun (2012) and Zhou *et al.* (2010)
miR171	GRAS transcription factors—response to abiotic stresses and floral development	*Ath*↑, *Tdi*↓, Rice↑↓, *Mtr*↓, *Ppe*↑, *Pto*↓,	Eldem *et al.* (2012), Kantar *et al.* (2011), Llave *et al.* (2002), Liu *et al.* (2008), Ren *et al.* (2012), Wang *et al.* (2011) and Zhou *et al.* (2010)
miR172	cDNA floral homeotic protein APETALA2, bZIP transcription factor family protein—flowering time, floral organ identity, cold stress response	*Ath*↑,Rice↓, *Pto*↑	Jones-Rhoades and Bartel (2004), Ren *et al.* (2012) and Zhou *et al.* (2010)
miR319	TCPcell differentiation, leaf development and biosynthesis of jasmonic acid	*Ath*↑, Rice↑↓, *Pto*↑	Efroni *et al.* (2008), Ren *et al.* (2012), Sarvepalli and Nath (2011), Schommer *et al.* (2008), Sunkar and Zhu (2004) and Zhou *et al.* (2010)
miR390	ARF—auxin-mediated transcriptional activation/suppression	*Pto*↓	Allen *et al.* (2005) and Ren *et al.* (2012)
miR393	TIR1 and AFB2 and AFB3—susceptibility to virulent bacteria	*Ath*↑ *Ppe*↓	Liu *et al.* (2008), Navarro *et al.* (2006) and Eldem *et al.* (2012)
miR394	Dehydration-responsive protein and F-box proteins—abiotic stress-response pathway	*Pto*↑, Ptc↓, *Gma*↑	Li *et al.* (2011a,b), Ren *et al.*(2012) and Shuai *et al.* (2013)
miR395	Sulphate transporter—response to sulphate deprivation	Rice↑, *Ppe*↓, *Pto*↓	Eldem *et al.* (2012), Liang *et al.* (2010), Ren *et al.* (2012) and Zhou *et al.* (2010)
miR396	GRL transcription factors; ceramidasegenes—leaf and cotyledon development	*Ath*↑ Rice↓ *Mtr*↓ *Ppe*↓	Eldem *et al.* (2012), Kantar *et al.* (2011), Liu *et al.* (2008), Liu and Yu (2009), Sun (2012), Wang *et al.* (2011) and Zhou *et al.* (2010)
miR397	Laccases—lignin biosynthesis, ion absorption and stress response	*Ath*↑, Rice↓, *Ppe*↓, *Pto*↓	Abdel-Ghany and Pilon (2008), Ding and Zhu (2009), Eldem *et al.* (2012), Ren *et al.*(2012), Sunkar and Zhu (2004) and Zhou *et al.* (2010)
miR398	Copper superoxide dismutases; cytochrome C oxidase subunit V—Copper	*Mtr*↑, *Tdi*↑, *Mtr*↓, *Ppe*↓	

Table 1 Continued

miRNA	Target name and functions*	Species[†]	Source
	homoeostasis, oxidative stress; enzyme involved in respiration		Eldem *et al.* (2012), Jones-Rhoades and Bartel (2004), Kantar *et al.* (2011), Sunkar *et al.* (2006), Trindade *et al.* (2010) and Wang *et al.* (2011)
miR399	Phosphate transporter–role in response to phosphate starvation	*Mtr*↑, *Pto*↑↓	Bari *et al.* (2006), Jones-Rhoades and Bartel (2004), Ren *et al.* (2012) and Wang *et al.* (2011)
miR403	AGO2—miRNA functioning	*Pto*↑	Allen *et al.* (2005) and Ren *et al.* (2012)
miR408	Chemocyanin precursor, cDNA phosphatidylinositol 3 and 4—kinase family protein, Peptide chain release factor—pollen tube growth	Rice↓, *Ath*↑, *Mtr*↑, *Ppe*↓, *Pto*↓, *Ptc*↓	Eldem *et al.* (2012), Liu *et al.* (2008), Ren *et al.* (2012), Shuai *et al.* (2013), Trindade *et al.* (2010) and Zhou *et al.* (2010)
miR474	Kinesin, a pentatricopeptide repeat (PPR) family protein-Motor functions; organelle biogenesis	Rice↑ *Tdi*↑	Kantar *et al.* (2011), Lu *et al.* (2005) and Zhou *et al.* (2010)
miR528	POD—Elimination of ROS	*Z. mays*↓	Wei *et al.* (2009)
miR827	NAD (P)-binding and SPX (SYG1/Pho81/XPR) proteins—activate in signal transduction pathways	*Z. mays*↑	M. Aukerman and W. Park (unpubl. data) and Zhang *et al.* (2009)
miR1432	Poly (ADP-ribose) polymerase; calcium-binding EF hand domains—activate in signal transduction pathways	*Tdi*↑	Kantar *et al.* (2011) and Zhang *et al.* (2009)
miR1444[‡]	Polyphenol oxydase—Probable role for improving plant water stress	*Ptc*↓	Khraiwesh *et al.* (2012), Shuai *et al.*, (2013) and Thipyapong *et al.* (2004)
miR2118	TIR-NBS-LRR domain protein—response to salinity, drought, cold and ABA stress	*Mtr*↑	Jagadeeswaran *et al.* (2009) and Wang *et al.* (2011)

*AFB, Auxin F-box protein; AGO2, Family member of ARGONAUT protein; AP2, APETALA2; ARF, auxin response factors; bHLH, basic helix–loop–helix; bZIP, Basic leucine zipper domain; CBF, CCAAT-binding factor; DCL1, Dicer Like1; GRAS, GAI, RGA, SCR; GRL, growth-regulating factor; GRML, Gibberellin response modulator-like protein; HD-ZIP, class III homeodomain leucine zipper; L-RTMK, Leucine-rich repeat transmembraneprotein kinase; MAPK, Mitogen-activated protein kinase; NAC domain TF, (NAM, ATAF1/2 and CUC2) domain proteins; NB-ARC domain protein, NB, ARC1 and ARC2 (functional ATPase domain—Probable regulation for activating the resistance proteins); NBS-LRR domain protein, Nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins; NF-YA, Nuclear factor Y subunit A; PDC, pyruvate decarboxylase isozyme1; PPRs, pentatricopeptide repeat (PPR) proteins; POX/POD, Peroxidase; SBP, Squamosa promoter-binding protein; SCL, scarecrow-like; SlMRP1, Multidrug resistance-associated protein gene; SNF7, Vacuolar-sorting protein; TCP-TEOSINTE BRANCHED/CYCLOIDEA/PCF) transcription factor genes; TF, transcription factor; TIR1, transport inhibitor response1.

[†]↑, up-regulation by drought; ↓, down-regulation drought; *Ath*, *Arabidopsis*; *Bdi*, *Brachypodium distachyon*; *Gma*, *Glycine max*; *Hvu*, *Hordeum vulgare*; *Mtr*, *Medicago truncatula*; *Peu*, *Populus euphratica*; *Ptc*, *Populus trichocarpa*; *Pto*, *Populus tomentosa*; *Ppe*, *Prunus persica*; *Tdi*, *Triticum dicoccoides*; *Z. mays*, *Zea mays*.

[‡]This miRNA was only found in *Populus trichocarpa*. All the rest miRNAs listed in Table 1 are present in more than three plant species and hence are considered as conserved miRNAs.

2008; Sunkar and Zhu, 2004). The up-regulated miRNAs were also shown to be involved in different developmental stages (Alonso-Peral *et al.*, 2012; Curaba *et al.*, 2013; Vaucheret *et al.*, 2006; Wu and Poethig, 2006; Wu *et al.*, 2006; Xie *et al.*, 2014; Zhu and Helliwell, 2011), suggesting that the regulation of drought tolerance and development by miRNAs is tightly linked, which probably undergoes via the same mechanism. It is very common that the expression level or drought responsiveness of a miRNA is species dependent (Arenas-Huertero *et al.*, 2009; Barrera-Figueroa *et al.*, 2012; Frazier *et al.*, 2011; Kantar *et al.*, 2011; Kulcheski *et al.*, 2011; Liu *et al.*, 2008; Lu *et al.*, 2008; Trindade *et al.*, 2010; Zhao *et al.*, 2007; Zhou *et al.*, 2010). For example, drought up-regulates miR156 in *Arabidopsis*, *Prunus persica*, barley, *Panicum virgatum* and *Triticum dicoccoides* (Eldem *et al.*, 2012; Kantar *et al.*, 2010, 2011; Sun *et al.*, 2012b; Sunkar and Zhu, 2004), but down-regulates it in rice and maize (Wei *et al.*, 2009; Zhou *et al.*, 2010). Similarly, drought stress down-regulates miR169 in *Arabidopsis*, *P. persica*, *P. virgatum* and *Medicago truncatula* (Li *et al.*, 2008), but up-regulates it in rice, *Glycine max*, *Populus euphratica* and tomato (Li *et al.*, 2011a,b; Qin *et al.*, 2011; Zhang *et al.*, 2011; Zhou *et al.*, 2010). miR1510 is up-regulated in *Glycine max* but down-regulated in *M. truncatula* and miR396 is down-regulated in *M. truncatula* and *Vigna unguiculata*, but up-regulated in *G. max* (Mantri *et al.*, 2013). In some plant species, members of the same families were found to be differently expressed under drought stress, for example, drought stress down- and up-regulates respective members of the miR319 family in rice (Zhou *et al.*, 2010). In fact, even the same miRNA in the same plant species can show different responses to drought depending on the exact conditions. For instance, in one study expression level of miR398a/b in *M. truncatula* was increased under drought stress (Trindade *et al.*, 2010), while in another study, expression level of the same miRNA in the same plant species decreased under drought stress (Wang *et al.*, 2011). Such differences may reflect different degrees of drought stress (Wang *et al.*, 2011) and high sensitivity of some miRNAs to subtle differences in growing conditions. Indeed, with different externally applied concentrations of polyethyleneglycol (PEG), a chemical that simulates drought conditions, the same miRNAs such as miR167, miR172, miR393, miR395, miR396, miR398 and miR399 in tobacco plants showed different degrees of up- or down-regulation (Frazier *et al.*, 2011). It is possible that differential expression of the same miRNA in the same plant species under drought conditions is the result of different spatial–temporal manner. It is likely that under drought conditions regulators of miRNA genes change their expression, which in turn leads to the change in expression of miRNAs and ultimately that of miRNAs' targets (Reyes and Chua, 2007; Trindade *et al.*, 2010). It is worth mentioning here that although miRNAs are conserved across different plant species, their targets may not be (Lu *et al.*, 2005). Therefore, the targets of miRNAs need to be identified in individual plant species. Target validation can also help provide functional evidence of the conserved and specific miRNAs in plant species.

miRNAs are also differentially expressed between different tissues or developmental stages under drought stress (Reinhart *et al.*, 2002). This has been the case for miR169, which in rice is induced more prominently in the roots than in the shoots. Members of the miR169 family are encoded by many loci within some plant species. However, in *Arabidopsis*, only miR169a and miR169c are substantially down-regulated by drought stress (Li *et al.*, 2008). Compared to other miR169 loci, the miR169a locus produces 90% of the total miR169 population, suggesting that miR169a would play a major role in response to drought stress. If this is true, then miR169 is likely to be regulated by drought stress at the transcriptional level at their loci. A previous study showed that transgenic overexpression of drought down-regulated miR169a and miR169c in *Arabidopsis* increased drought sensitivity of the plants (Li *et al.*, 2008). However, two members of the miR169 family in rice, miR169g and miR169n/o and one member in tomato, miR169c, were up-regulated by drought stress (Zhang *et al.*, 2011; Zhao *et al.*, 2009). In addition, overexpression of miR169c in tomato reduced stomatal conductance and water loss compared to nontransgenic tomato and hence enhanced drought tolerance (Zhang *et al.*, 2011). These differences in outcomes of overexpressing miR169c in different plant species have been suggested to be caused by different timing, duration and intensity of the stress that was applied in the different studies (Covarrubias and Reyes, 2010). It is likely that the level of miRNA169 could vary during the course of the stress treatment. This has been the case for miR398 in *Arabidopsis* (Jia *et al.*, 2009). The reduced accumulation of miRNAs under drought could be because of interference with their biogenesis pathway (Covarrubias and Reyes, 2010). The contribution of miR169 to drought tolerance or intolerance could depend on its promoter because two dehydration-responsive elements (DREs) were identified in the promoter of MIR169g (Zhao *et al.*, 2007). Further studies showed that miR169 targets nuclear factor Y (NF-Y) transcription factor (TF), known as a heme-activated protein (HAP) or CCAAT-binding factor (CBF), by reducing the NF-Y mRNA level (Li *et al.*, 2008). Furthermore, transgenic overexpression of NFYA5, a subunit of the NF-Y TF, has been shown to increase drought tolerance (Li *et al.*, 2008). These pieces of evidence indicate that the contribution of miR169 to drought tolerance is via the NF-Y TF and that the down-regulation of miR169 contributes to the high level of NFYA5 observed under drought stress.

Transgenic overexpression of osa-miR319 in creeping bentgrass and of miR394 in soya bean also increased drought tolerance (Ni *et al.*, 2012; Zhou *et al.*, 2013). Both miRNAs are up-regulated in most plant species under drought stress (Ni *et al.*, 2012; Zhou *et al.*, 2010). However, under other stress conditions such as salinity, cadmium toxicity or low iron and sulphate, the regulation of miR394 shows differences between plant species (Huang *et al.*, 2010; Kong and Yang, 2010). One of the miR394's targets has been identified to encode an F-box protein (At1g27340) involved in the regulation of leaf curling-related morphology in *Arabidopsis* (Song *et al.*, 2012). miR393 is another key miRNA for the regulation of the F-box genes in many plant species including *Arabidopsis*, rice, *M. truncatula*, *Pinguicula vulgaris* and sugarcane (Ferreira *et al.*, 2012), whose expression is altered by drought stress. Like miR394, miR393 is up-regulated by drought stress in most plant species and is responsive to other abiotic stresses such as salinity, low temperature and aluminium toxicity (Arenas-Huertero *et al.*, 2009; Liu *et al.*, 2008; Sunkar and Zhu, 2004; Trindade *et al.*, 2010; Zhao *et al.*, 2007). Transgenic overexpression of miR393 in rice increased salinity tolerance, suggesting the native gene may regulate salinity tolerance (Gao *et al.*, 2011). Transgenic overexpression of miR393 in rice resulted in hyposensitivity to synthetic auxin analogue treatments (Xia *et al.*, 2012), suggesting that native miR393 may regulate auxin signalling and would thus reduce plant growth under drought stress. Under drought, endogenous concentrations of auxin, gibberellin and cytokinin usually decrease, whereas ABA and ethylene increase (Nilsen and Orcutte, 1996). In line with this hypothesis, miR393 was found to target transport inhibitor response 1 (TIR1), known as an auxin receptor and positive regulator of auxin signalling that acts via degradation of Aux/IAA proteins (Dharmasiri and Estelle, 2002; Windels and Vazquez, 2011). However, how miR393 regulates its targets remains unclear. Table 2 summarizes studies in which transgenic alteration of miRNA expression was tested for effects on drought tolerance.

A number of legume-specific miRNAs were identified in *Phaseolus vulgaris* plants treated by drought and ABA, and targets of these miRNAs were annotated to be involved in diverse cellular processes unique to legumes (Arenas-Huertero *et al.*, 2009). Using deep sequencing technology, Kulcheski *et al.* (2011) identified 256 miRNAs from genotypes of soya bean that were susceptible or resistant to drought or rust. Of these miRNAs, 71 belonged to conserved miRNA soya bean families, while 15 miRNAs belonging to six families were conserved in other plant species. Twenty-nine miRNAs belonging to 24 novel families were reported for the first time in soya bean. The authors also reported 121 alternative isoforms (miRNA variants) derived from 22 conserved miRNA families and four novel miRNA families. An interesting point is that among 11 miRNAs analysed, all were expressed differently from each other during drought stress. However, the majority were up-regulated in a susceptible genotype but down-regulated in a tolerant genotype under drought. This distinct miRNA behaviour across the two genotypes may reflect regulation of the genes associated with drought stress tolerance or intolerance. Similarly, Barrera-Figueroa *et al.* (2011) used deep sequencing of sRNA libraries from two cowpea genotypes (drought tolerant and susceptible) to identify 157 miRNAs which belonged to 89 families. Forty-four drought-responsive miRNAs belonging to 28 families were identified by comparing expression levels in stressed versus control plants. Of them, 30 miRNAs were up-regulated while 14 miRNAs were down-regulated. These drought-responsive miRNAs included miRNA families which were already known to be drought-

Table 2 miRNA transgenics for drought tolerance

Overexpressed miRNA	Species	Transgenic plants exhibited	Possible mechanism	References
miR164	*Arabidopsis*	Leaf longevity	Ethylene signalling molecule, EIN2	Kim *et al.* (2009)
miR169c	Tomato	Reduced stomatal conductance and transpiration rate	Unknown	Zhang *et al.* (2011)
miR169a	*Arabidopsis*	Increased leaf water loss and greater sensitivity to drought stress	Unknown	Li *et al.* (2008)
Osa-miR319	Creeping bentgrass	Increased leaf wax content and water retention capacity	Unknown	Zhou *et al.* (2013)
miR393	Rice	Increased tillering, early flowering and reduced tolerance to salt and drought	Hyposensitivity to auxin	Xia *et al.* (2012)
Gma-miR394a	*Arabidopsis*	Recovery from drought stress	Possible involvement of F-box proteins in abiotic stress-response pathway	Ni *et al.* (2012)

responsive in other plant species, indicating that these miRNA families may be involved in conserved drought-response pathways. In addition, predicted target genes of 32 miRNAs were shown to have diverse predicted physiological functions. Most of these predicted targets were TFs.

Drought up- or down-regulated miRNAs are both potentially relevant for engineering plant drought tolerance, as miRNA targets probably include genes that contribute both positively or negatively to tolerance. The up-regulation of miRNAs means that their targets are down-regulated under the same conditions and vice versa. Enhancing the accumulation of target(s) contributing to drought tolerance could be achieved either by overexpressing target genes, or by silencing the corresponding miRNA (Sunkar *et al.*, 2007). For example, down-regulated miR168 and miR528 under drought stress resulted in accumulation of their targets, mitogen-activated protein kinase (MAPK) and peroxidase (POD) (Wei *et al.*, 2009). In this experiment, ABA levels significantly increased in maize tissues, which in turn enhanced the formation of reactive oxygen species (ROS), which further up-regulated MAPK for inducing the expression of antioxidant genes and antioxidant enzymes. Both ABA and ROS are important signalling molecules that regulate many developmental processes and stress-adaptive processes in plants (Cutler *et al.*, 2010). Antioxidant enzymes also limit ROS levels to help achieve drought stress. Likewise, an increased level of POD also results in the elimination of ROS and alleviation of drought injury (Wei *et al.*, 2009). Therefore, the down-regulation of miR168 and miR528 under drought stress is expected to increase drought tolerance. A study conducted by Shuai *et al.* (2013) showed that the down-regulation of miR160 and miR164 in drought-stressed *P. trichocarpa* also allows increased expression of their targets, ARF and NAC domain TFs. Overexpression of these TFs in rice has been shown to enhance drought stress tolerance in the field under severe drought stress conditions at the reproductive stage (Hu *et al.*, 2006) as well as at the seed germination and postgermination stages (Liu *et al.*, 2007). Drought down-regulated miRNAs in *P. trichocarpa* also included miR408, miR1444 and miR394, which target dehydration-responsive proteins such as early responsive dehydration-related protein (ERD) and polyphenol oxidase (PPO) (Shuai *et al.*, 2013). Increased expression levels of these targets help lessen drought injury in transgenic plants (Shuai *et al.*, 2013). In the same study, two novel miRNAs Ptc-miRn6 and Ptc-miRn16 were also confirmed to be down-regulated in *P. trichocarpa*, but the function of the targets of these two miRNAs are unknown (Shuai *et al.*, 2013). Therefore, alteration in miRNA profiles seems to play crucial roles in attenuating plant growth and development under stresses. In a nutshell, these findings highlight the importance of detailed characterization of stress-responsive miRNAs in plants.

Drought-responsive miRNAs in wheat and barley

Wheat and barley are two of the most important cereals in the world and are crops that are seriously affected by drought. Furthermore, both *Triticeae* species contain large and repetitive genomes, which are, respectively, much larger than that of rice or *Arabidopsis*. Therefore, in this section, we particularly summarize the recent knowledge on drought-responsive miRNAs in these two crops, which were as yet given little attention before.

In miRBase (Release 20: June 2013), 69 miRNAs from barley and 43 miRNAs from wheat were described. Additional barley and wheat miRNAs were described in published papers (Colaiacovo *et al.*, 2010; Curaba *et al.*, 2012; Dryanova *et al.*, 2008; Hackenberg *et al.*, 2012a,b, 2014; Han *et al.*, 2014; Jin *et al.*, 2008; Kantar *et al.*, 2010, 2011; Li *et al.*, 2013; Lucas and Budak, 2012; Lv *et al.*, 2012; Meng *et al.*, 2013; Schreiber *et al.*, 2011; Sun *et al.*, 2014; Wang *et al.*, 2014; Wei *et al.*, 2009; Xin *et al.*, 2010; Yao *et al.*, 2007, 2010). Barley miRNAs were initially predicted from available barley EST sequences by Dryanova *et al.* (2008). These included 28 conserved miRNAs belonging to 15 miRNA families. A more sophisticated computational prediction approach was then used to extend this to 156 miRNAs belonging to 50 miRNA families (Colaiacovo *et al.*, 2010). In 2011, 100 barley miRNAs were experimentally identified by deep sequencing small RNAs of barley cultivar Golden Promise (Schreiber *et al.*, 2011). Of these miRNAs, 56 were shown to be expressed as orthologs in other species, while 44 miRNAs were known to be expressed only in barley. Soon after, deep sequencing of small RNAs from a different barley cultivar, clipper, identified 259 miRNAs, of which133 were novel (Lv *et al.*, 2012). Using psRNA target, a plant small RNA target analysis server (http://plantgrn.noble.org/psRNATarget/), 267 targets of barley miRNAs were predicted (Lv *et al.*, 2012). These targets were predicted to be involved in many developmental processes such as seed germination, vegetative and reproductive phase changes, flowering initiation and seed production (Lv *et al.*, 2012). However, the validation of these miRNA targets is likely to be complex as each miRNA may control many genes and each gene can be controlled by many miRNAs (Yang and Qu, 2013).

Wheat miRNAs were first computationally predicted in 2005 (Zhang *et al.*, 2005), at which time only 16 miRNAs belonging to nine conserved miRNA families were identified from wheat EST databases. In 2007, 58 miRNAs belonging to 43 miRNA families were discovered by cloning and sequencing of wheat small RNAs (Yao *et al.*, 2007). So far, 270 known miRNAs have been reported in wheat (Dryanova *et al.*, 2008; Jin *et al.*, 2008; Kantar *et al.*, 2010, 2011; Lucas and Budak, 2012; Pandey *et al.*, 2014; Wei *et al.*, 2009; Xin *et al.*, 2010). The identified barley and wheat miRNAs provide a platform for further analysis of expression profiles of miRNAs and characterization of drought-responsive miRNAs in barley and wheat.

Of the miRNAs identified in barley and wheat, relatively few are drought responsive. Of 28 miRNAs in barley studied by Kantar *et al.* (2010), only four (hvu-miR156a, hvu-miR166, hvu-miR171 and hvu-miR408) were found to be differentially expressed under dehydration stress conditions. All four dehydration-regulated miRNAs were found to be induced by drought in barley leaves (Kantar *et al.*, 2010). By contrast, in barley roots, hvu-miR166 expression was suppressed by drought and the expression of the other three miRNAs was unchanged by it (Kantar *et al.*, 2010). As expected, the targets were found to be inversely expressed relative to the respective miRNAs in these tissues, with the exception of miR408's target, whose expression could not be detected in leaf (Kantar *et al.*, 2010). Later, a further three conserved miRNAs (miR156d miR396d and miR399b) and three novel miRNAs (miR-n026a*, miR-n029 and miR-n035) were found to be up-regulated under drought in barley leaves (Lv *et al.*, 2012). The three novel miRNAs were also shown to be up-regulated by salinity in barley leaves (Lv *et al.*, 2012). Very recently, 31 barley miRNAs were detected in barley cv. Golden Promise treated by drought, of which 13 were significantly down-regulated, while one miRNA (hvu-miR5049b) was significantly up-regulated, under the drought conditions (Hackenberg *et al.*, 2014). Hvu-miR399 was not expressed under drought (Hackenberg *et al.*, 2014), indicating that the expression of this miRNA may be drought dependent. Of 74 conserved miRNAs detected in Golden Promise, 20 belonging to ten miRNA families were significantly drought down-regulated, while one miRNA (gma-miR6300) was significantly up-regulated (Hackenberg *et al.*, 2014). However, some drought-regulated miRNAs were inconsistently expressed across different barley tissues. Moderately expressed hvu-miR166a was drought up-regulated in barley leaves but down-regulated in roots. Hvu-miR168-5p was only drought up-regulated in leaves while in root tissues its expression level was unchanged. Osa-miR393a and hvu-miRX35 were expressed in leaf but not in root (Hackenberg *et al.*, 2014). All the drought-regulated miRNAs detected in Golden Promise showed expression patterns that were similar to those reported for the corresponding miRNAs in other barley cultivars, under the same drought conditions, as judged by Northern hybridization or quantitative real-time reverse transcription PCR (qRT-PCR) (Hackenberg *et al.*, 2014). The regulation of these miRNAs by drought may be partly associated with drought-related TFs such as DREB TFs (Hackenberg *et al.*, 2012a; Morran *et al.*, 2011).

Drought-regulated miRNAs were identified by the micro-array approach from a wild wheat, *T. turgidum* ssp. *dicoccoides* (Kantar et al. (2011). At 4 and 8 h postdrought treatment, 438 miRNAs were identified in leaf and root tissues while in control plants only 205 miRNAs were detected (Kantar *et al.*, 2011). A comparison showed that 13 miRNAs (miR1867, miR896, miR398, miR528, miR474, miR1450, miR396, miR1881, miR894, miR156, miR1432, miR166 and miR171) were differentially expressed between the drought and water conditions (Kantar *et al.*, 2011). However, none of these miRNAs have their targets experimentally validated (Kantar *et al.*, 2011). miR1450 was drought up-regulated in *T. dicoccoides* (Kantar *et al.*, 2011), but drought down-regulated in *P. trichocarpa* (Lu *et al.*, 2008). miR1450 was also down-regulated in *P. trichocarpa* by saline conditions (Lu *et al.*, 2008). The results for miR1450 suggest that this miRNA may be controlled by different regulatory networks in different plant species. Transgenic rice overexpressing miR159 from wheat was found to be more sensitive to heat stress, indicating that miR159 might participate in a heat stress-related signalling pathway and influence heat stress tolerance (Wang *et al.*, 2012). Intriguingly, the transgenic rice also delayed heading and increased male sterility (Wang *et al.*, 2012). Targets of miR159 were identified as *MYB33* and *MYB101* which are important players in responses to ABA accumulation under drought stress (Reyes and Chua, 2007). Taken together, these data indicate that drought-responsive miRNAs can be used as a tool in the genetic modification for future improvement of cereal crops tolerant to drought.

Mechanisms of drought stress responses of miRNAs

Abscisic acid-responsive elements in miRNA genes

Suppression of lateral root growth by drought stress has been widely accepted as an adaptive response, because it allows redirection of resources towards production of deeper roots, enabling more efficient extraction of water from deep in the soil. Epoxycarotenoid cleavage-derived ABA has been shown to serve as a specific stress signal in plants (Nambara and Marion-Poll, 2005). Under drought stress, ABA is formed in the dehydrating roots, which inhibits lateral root growth (Xiong *et al.*, 2006). miRNA393 was found to be strongly up-regulated by ABA (Sunkar and Zhu, 2004). An ABA hypersensitive mutant of *A. thaliana (fry 1)* was shown to reduce lateral root growth at an elevated miR393 level (Chen *et al.*, 2012). Hence, miR393 was proposed to be a regulator of root adaptation under drought stress. Known targets of miR393, two auxin receptors (TIR1 and AFB2), undergo post-transcriptional silencing through miR393-guided cleavage—a process that is required for drought inhibited induced lateral root growth (Chen *et al.*, 2012). Promoters of most ABA-responsive genes have a conserved cis element as well as ABA-response elements (ABREs), which have been considered to play a role in stress-responsive expression (Mundy *et al.*, 1990; Xu *et al.*, 1996). In *Arabidopsis*, miR167 is up-regulated under drought stress and has ABREs in the promoter of the corresponding gene (Liu *et al.*, 2008). miR167 targets two auxin response factors (ARFs) that play a role in root architecture (Wu *et al.*, 2006). In this regard, miRNA393 and miR167 seem like good candidate miRNAs for use in studying drought-adaptive mechanisms.

Abscisic acid is also known to play an important role in seed dormancy. It is responsible for the instigation and maintenance of dormancy (Rodriguez-Gacio *et al.*, 2009). Reyes and Chua (2007) showed that in *Arabidopsis* miR159 levels increased with the addition of exogenous ABA or under drought treatment during seed germination (Reyes and Chua, 2007). miRNA159 mediates cleavage of MYB101 and MYB33 transcripts that function as positive regulators of ABA responses in the plants (Reyes and Chua, 2007). In support, overexpression miR159 suppresses MYB33 and MYB101 transcript levels in the transgenic plants and renders the plants hyposensitive to ABA (Reyes and Chua, 2007). Consistent with this, transgenic plants overexpressing cleavage-resistant forms of MYB33 and MYB101 are also hypersensitive to ABA (Reyes and Chua, 2007). By facilitating seed dormancy under stress (when endogenous ABA is high), the target mRNA of miR159 might therefore have a crucial role in ensuring avoidance to drought. Recent studies showed that drought and ABA up-regulated miRNAs also include miR169, miR319, miR397, miR2118, miR393 and miR167 (Khraiwesh *et al.*, 2012). However, it is unknown whether the promoters of these miRNA genes contain ABRE cis elements that are important for abiotic stress responses (Mundy *et al.*, 1990; Xu *et al.*, 1996). miR168 and miR396 contain the ABRE cis elements in their promoter regions and up-regulated by drought stress (Liu *et al.*, 2008). ABRE elements present in the promoter regions of miRNA genes could influence drought tolerance mechanisms.

Ethylene signalling and regulation of miRNAs

miRNAs could influence leaf senescence (Lim *et al.*, 2007). Leaf senescence is regarded as a drought avoidance mechanism, as it can reduce canopy size and transpiration, and allow remobilization of water and nutrients to organs more crucial for survival and reproduction of the plant (Griffiths *et al.*, 2014). In the study by Kim *et al.* (2009), miR164 was proposed to be a regulator of leaf senescence in *Arabidopsis*, based on the fact that *EIN2* (ETHYLENE INSENSITIVE 2), an ethylene signalling protein in *Arabidopsis*, down-regulates miR164 in older leaves. This results in increasing levels of its targets *NAC1*, *ORE1* and At5g61430. Accordingly, miR164 overexpression and/or lack of its target *ORE1* activity resulted in enhanced leaf longevity. The study further indicated that Ath-miR164 negatively regulates cell death and senescence in younger leaves through down-regulation of *ORE1* (Kim *et al.*, 2009). Drought stress triggers ethylene production in higher plants, which in turn enhances leaf senescence (Apelbaum and Yang, 1981; McKeon *et al.*, 1982; McMichael *et al.*, 1972) indicating a further link of the aforementioned pathway to drought adaptation.

In drought-resistant wild emmer wheat, miR166 was shown to be down-regulated by drought stress (Kantar *et al.*, 2011). Expression of miR166 is also regulated by two members of the GRAS family of TFs, SHORT-ROOT (SHR) and SCARECROW (SCR). SHR and SCR are both sensitive to ABA (Cui *et al.*, 2012). miR166 activated by SHR and SCR in turn down-regulates the HD-Zip TFs (Carlsbecker *et al.*, 2010; Miyashima *et al.*, 2011; Williams *et al.*, 2005). By contrast, the HD-Zip TF-encoded gene *Hahb-4* was up-regulated under drought stress and ABA treatments (Dezar *et al.*, 2005). An ethylene-responsive element was found in the promoter region of *Hahb-4* (Manavella *et al.*, 2006). Correspondingly, in *Arabidopsis*, *Hahb-4* was found to be up-regulated during ethylene-mediated leaf senescence and transgenic overexpression of this gene enhanced drought tolerance (Manavella *et al.*, 2006). However, it is unclear how miR166 regulates HD-Zip TFs and what genes are regulated by HD-Zip TFs.

Other drought-inducible promoter elements in MIR genes

According to the unpublished data of M. Aukerman and W. Park, up-regulated miR827 is considered to be necessary for drought tolerance in maize. miR399 and miR2111 have also been reported to be up-regulated in *M. truncatula* under drought stress (Wang *et al.*, 2011). These three miRNAs are reported to be up-regulated by phosphate starvation in *Arabidopsis* (Bari *et al.*, 2006; Hackenberg *et al.*, 2012b; Hsieh *et al.*, 2009; Pant *et al.*, 2008). Previously, a member of the MYB TF super family was found to be involved in phosphate starvation signalling (Rubio *et al.*, 2001). On the other hand, MYB TF-binding sites are reported to be drought-inducible promoter elements in *Arabidopsis* (Liu *et al.*, 2008). It is possible that the MYB TF promoter-binding sites facilitate both drought and phosphate starvation-induced expression of MIR genes. In comparison of *Arabidopsis* and rice, little is known about cis-regulatory elements in the promoters of miRNA genes in barley and wheat.

Strategies for functional analysis of miRNAs and their targets in plants

miRNAs are negative regulators of genes. Their short sequence length makes it relatively easy for them to base pair with other sequences, potentially allowing regulation of multiple genes. This, combined with the existence of gene–gene interaction networks, makes the biological implications of miRNA action difficult to ascertain. Generally, two transgenic strategies can be adopted to determine the functions of miRNAs. One is to use gain of function to increase miRNA expression and the other is loss of function to reduce or abolish miRNA expression. Gain of function can be achieved by overexpressing the miRNA, using a constitutive promoter such as the 35S or polyubiquitin promoters, or an inducible promoter that is activated only under certain conditions. Loss of function can be accomplished by overexpressing antisense miRNAs. Antisense miRNAs inactivate miRNA activities by base pairing with miRNAs and have been widely used in the functional analysis of animal miRNAs (Thomson *et al.*, 2011). Artificial miRNAs (amiRNAs), generated by replacing the miRNA duplex regions in native miRNA precursors, can be used to achieve either gain or loss of function (Ossowski *et al.*, 2008). Compared to miRNAs and antisense miRNAs, amiRNA sequences can be optimized for high efficiency because they are generated from the same locus in their precursors (Warthmann *et al.*, 2008). A website is currently available for the automated design of amiRNAs (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi?page=Home;project=stdwmd).

If miRNA's targets are known, then miRNA functions can be analysed by modulating the expression of the targets. The knockdown or abolishment of the transcription of miRNA's targets can be achieved using amiRNAs, which can specifically silence single or multiple genes of interest (Alvarez *et al.*, 2006; Duan *et al.*, 2008; Khraiwesh *et al.*, 2008; Molnar *et al.*, 2009; Ossowski *et al.*, 2008; Schwab *et al.*, 2006; Warthmann *et al.*, 2008). Unlike antisense, the amiRNA sequence does not have to be perfectly complementary to the target sequence (Schwab *et al.*, 2006; Warthmann *et al.*, 2008). Therefore, specific nucleotides within the amiRNAs can be optimized to particular gene(s), which do not affect the pre-miRNA processing and the biogenesis of mature miRNAs (Niu *et al.*, 2006; Vaucheret *et al.*, 2004; Warthmann *et al.*, 2008; Zeng *et al.*, 2002) and result in more accurate gene silencing (Duan *et al.*, 2008; Park *et al.*, 2009; Tang, 2010). miRNA mimics are another way to analyse functions of both miRNAs and their targets in both plants and animals (Franco-Zorrilla *et al.*, 2007; Thomson *et al.*, 2011). miRNA mimics can be designed to target gene promoters, and these have been shown to work in human cells (Place *et al.*, 2008). Different from endogenous miRNAs, miRNA mimics act in a gene-specific manner. Either miRNA mimics, antisense miRNAs or amiRNAs can be used in transient assays for quickly examining the expression relationship between miRNAs and their targets (Johansen and Carrington, 2001).

Conclusion and future directions

Changing climate, variable weather patterns and other environmental stresses are a matter of concern for agricultural crop production. Drought is a stress limiting crop production and yield across the world. Drought tolerance is a complex trait involving a number of gene regulatory networks that miRNAs participate in. However, the mechanisms of miRNAs involvement in stress tolerance and their target regulatory networks are not well understood. This is partly due to the possibility of each endogenous miRNA regulating multiple genes and each gene being regulated by multiple miRNAs. Therefore, although many miRNAs have been identified from a variety of plants, some of which are shown to be drought regulated, the targets of these miRNA are still largely unknown. Hence, the major challenge ahead will be to discover the miRNA targets and how miRNAs function on the targets. This information will allow the identification of miRNAs/targets that influence drought tolerance. The other challenge will be to characterize the cis-regulatory elements in the miRNAs genes, to determine the corresponding TFs and to describe how the miRNAs are regulated by drought. These data would offer new insights for understanding the action of miRNAs and their potential to be used to engineer enhanced drought stress tolerance.

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