Review TRENDS in Plant Science Vol.12 No.7

Small RNAs as big players in plant abiotic stress responses and nutrient deprivation

Ramanjulu Sunkar[1], Viswanathan Chinnusamy[2], Jianhua Zhu[3] and Jian-Kang Zhu[3]

[1] Department of Biochemistry and Molecular Biology, 246 Noble Research Center, Oklahoma State University, Stillwater, OK 74078, USA
[2] Water Technology Centre, Indian Agricultural Research Institute, New Delhi, India
[3] Department of Botany and Plant Sciences and Center for Plant Cell Biology, 2150 Batchelor Hall, University of California, Riverside, CA 92521, USA

Abiotic stress is one of the primary causes of crop losses worldwide. Much progress has been made in unraveling the complex stress response mechanisms, particularly in the identification of stress responsive protein-coding genes. In addition to protein coding genes, recently discovered microRNAs (miRNAs) and endogenous small interfering RNAs (siRNAs) have emerged as important players in plant stress responses. Initial clues suggesting that small RNAs are involved in plant stress responses stem from studies showing stress regulation of miRNAs and endogenous siRNAs, as well as from target predictions for some miRNAs. Subsequent studies have demonstrated an important functional role for these small RNAs in abiotic stress responses. This review focuses on recent advances, with emphasis on integration of small RNAs in stress regulatory networks.

Small RNAs are ubiquitous regulators of gene expression

Plant development, metabolism and stress responses, as well as a myriad of other functions, depend on the correct regulation of gene expression. This is achieved by multiple mechanisms, with perhaps the most important control being exerted at the level of transcription. However, post-transcriptional events also play a crucial role in regulating gene expression. The stability of mRNAs is regulated by a variety of signals acting on specific sequences within the RNAs. This regulation is often mediated by specific RNA-binding proteins (RBPs) that bind to elements in the untranslated regions (UTRs) of mRNAs and regulate the stability, translation or localization of the mRNA [1–3]. The recent discovery of microRNAs (miRNAs, see Glossary) and small interfering RNAs (siRNAs) revealed another ubiquitous mode of post-transcriptional regulation. These small RNAs are known to silence genes post-transcriptionally by guiding target mRNAs for degradation or by repressing translation [4–8]. The role of miRNAs in controlling developmental processes has been at the forefront of plant miRNA research. This is based in part on the fact that many proteins (*dcl1*, *hen1*, *hyl*, *se* and *hst*) required for miRNA generation and miRNA target genes (*phb* and *ago1*) were first identified through genetic screens for developmental defects [9–15]. A comprehensive examination of miRNAs in plant development is provided by several excellent recent reviews [5–8,16–18]. In this review, the emerging roles of miRNAs and endogenous siRNAs in plant stress responses are discussed.

Overview of the role of miRNAs in plant stress responses

Plants are sessile organisms that must endure stressful environments. A large proportion of plant genes are regulated by stresses such as drought, soil salinity and extreme temperatures [19–23]. Of the many gene regulatory mechanisms such as transcriptional, post-transcriptional and post-translational regulation, transcriptional regulation is the most widely studied mechanism. The action of specific transcription factors that bind to conserved *cis*-acting promoter elements is well documented as a cause of changes in gene expression, particularly those induced by abiotic stress [20]. Furthermore, post-transcriptional gene regulation under stress conditions has been documented before, although the underlying mechanism was not known [24–26]. Considering the important roles of small RNAs in guiding post-transcriptional gene silencing, their involvement in stress-regulated gene expression seemed likely [27,28]. The discovery that stress can regulate miRNA levels, coupled with the identification of stress-associated genes as miRNA targets provided clues about the role of miRNAs in stress responses. Functional analyses have demonstrated that several plant miRNAs play vital roles in plant resistance to abiotic as well as biotic stresses [29–34]. Understanding small RNA-guided stress regulatory networks should provide new tools for the genetic improvement of plant stress tolerance (Figure 1). Indeed, it has been shown recently that manipulation of miRNA-guided gene regulation can help to engineer plants that will be more stress-resistant [34].

miRNA and oxidative stress

Under normal conditions, plants maintain a delicate balance between reactive oxygen species (ROS) production

Corresponding authors: Sunkar, R. (rsunkar@biochem.okstate.edu); Zhu, J.-K. (jian-kang.zhu@ucr.edu).
Available online 18 June 2007.

 1360-1385/$ – see front matter doi:10.1016/j.tplants.2007.05.001

Glossary

Argonaute proteins: Argonaute proteins are defined by the presence of PAZ and PIWI domains with slicer activity. The PAZ domain interacts with the 3′ end of single-stranded miRNA or siRNA and orienting and recognition of cleavage on the mRNA substrate. The PIWI (name originates from its initial finding in Drosophila, *P* element induced wimpy testis) domain is similar to an RNase H domain possessing slicer activity. Recent studies indicated that PAZ domains of Argonaute proteins interact directly with the 3′ ends of the small RNAs and PIWI, and the middle domains of Argonaute proteins with the 5′ end of small RNAs.

ASRP (*Arabidopsis thaliana* small RNA project, http://asrp.cgrb.oregonstate.edu/): a public resource developed exclusively for the *Arabidopsis* small RNAs by James Carrington's laboratory. *Arabidopsis* small RNA sequencing data is deposited in the database.

Dicer-like proteins: Dicer-like proteins are a class of RNase III endoribonucleases with two RNase III domains and a PAZ (Piwi Argonaut and Zwille) domain. The PAZ domain is an RNA-binding module found in Dicer-like proteins as well as Argonaute (Ago) proteins. Dicer excise the 20–24-nt small RNA duplexes with 2-nt 3′ overhangs, each strand bearing 5′ phosphate and 3′ hydroxyl termini either from single-stranded RNA adopting imperfect hairpin structure (primary miRNA transcript) or from long perfect dsRNAs formed as a result of RNA-dependent RNA polymerase activity or read-through transcription of inverted repeats or NAT pairs.

MicroRNA: the term short-temporal RNA (stRNA) had been used when this class of small RNA in *Caenorhabditis elegans* was described in 1993 [90,91], but the term microRNA (miRNA) was only coined in 2001. MicroRNAs are genome-encoded ~20–24-nucleotide (nt) duplex-structured small RNAs with 5′-phosphate and 3′-hydroxyl groups with 2-nt overhangs. These are excised by the cropping activity of Dicer-like 1 (RNase III-like endoribonucleases) on the primary miRNA transcript (pri-miRNA) that can adopt an imperfect hairpin-like structure. One of the duplexes serves as a guide strand, which is referred to as miRNA (and the antisense strand to miRNA is known as miRNA*), and is selectively loaded onto RISC and serves as a sequence-specificity determinant in recognizing the target transcript. Based on complete or partial complementarity between miRNA and its target transcript, RISC can cause either target mRNA degradation or inhibit protein synthesis. Some of these miRNAs are evolutionarily deeply conserved whereas some others are lineage-specific and even species-specific.

MPSS (massively parallel signature sequencing, http://mpss.udel.edu/at/): a sequencing-based technology that identifies short sequence signatures produced from a defined position within an mRNA. A modified version of MPSS has been adapted to sequence plant small RNAs.

NATsiRNA: a class of endogenous siRNAs derived from dsRNAs formed by annealing of sense and antisense transcripts encoded by natural *cis*-antisense gene pairs. NATsiRNAs are capable of regulating target mRNA expression at the post-transcriptional levels by guiding mRNA cleavage.

Natural-antisense transcripts (NATs): NATS formed from partial or complete overlapping genes on opposite strands of DNA from the same locus (*cis*-NATs) or from transcripts of distinct loci separate from their sense partners (*trans*-NATs).

RISC (RNA-induced silencing complex): RISC is a cytosolic complex with the Argonaute protein as a slicer and a 21-nt miRNA or siRNA as a guide molecule that can guide post-transcriptional gene silencing of the target transcript either by target mRNA degradation or by inhibiting the translation.

RITS (RNA-induced transcriptional silencing complex): an active RITS complex containing Argonaute protein and a 24-nt siRNA serving as a guide molecule. RITS complex functions in the nucleus and has the ability to cause DNA and histone modification leading to transcriptional gene silencing.

siRNA: 'small interfering RNA' or 'short interfering RNA' that can be derived from the genome (as shown in plants) or exogenously supplied in the form of a long duplex of nucleic acids. siRNAs are a class of ~20–24-nt small regulatory RNAs generated by the activity of a Dicer-like family of ribonucleases (DCL2, DCL3 and DCL4 in *Arabidopsis*) acting on a long double-stranded RNA with perfect duplex structure. Upon processing, only the guide strand is loaded onto the RISC or RITS complex. RISC or RITS loaded with the siRNA is capable of recognizing homologous RNA or DNA sequences and function in silencing the gene expression either at the post-transcriptional level (PTGS) or at the transcriptional level (TGS). Both PTGS and TGS pathways are thought to be the genome surveillance mechanisms providing protection against viral infections, transposon proliferations or introgression of aberrant transgenes. Endogenous siRNAs have been classified into at least three subclasses: repeat-associated siRNAs (rasiRNAs), *trans*-acting siRNAs (ta-siRNAs), and siRNAs derived from natural antisense transcripts (nat-siRNAs).

***trans*-acting short interfering RNAs (tasiRNA):** tasiRNAs form a class of 21-nt regulatory small RNAs found only in plants. tasiRNAs are generated from non-coding RNA precursors that are initially targeted for cleavage by a miRNA. RNA-dependent RNA polymerase acts on the cleavage products and converts them into dsRNAs that are cleaved into 21-nt tasiRNAs. Thus, the accumulation of tasiRNAs is dependant on the components of both miRNA (Dicer-Like1, Argonaute1, HYL1 and HEN1) and siRNA pathways (RNA-dependent RNA polymerase 6 and DCL4). tasiRNAs can guide cleavage of target mRNAs and regulate gene expression at the post-transcriptional level like plant miRNAs.

and scavenging. Plants have developed a highly sophisticated and efficient antioxidant system [20,22,35,36]. However, exposure to stress conditions such as drought, cold, salinity, high light and heavy metals results in the accumulation of excess ROS in plant cells. Superoxide radicals (O_2^-) are the primary products of photo-reduction of dioxygen in Photosystem I (PSI) of chloroplasts. These reactive O_2^- radicals need to be scavenged at the site of their synthesis to limit the generation of more toxic hydroxyl radicals. Cu-Zn superoxide dismutase 2 (CSD2) is attached to the thylakoid of chloroplasts, which is also the site of superoxide generation and, thus, plays an important role in localized and immediate scavenging of superoxide radicals. *SOD* genes are induced under oxidative stress to meet the requirement for superoxide detoxification [37,38]. Nuclear run-on assays indicate that *CSD2* as well as *CSD1* transcripts are not induced at the transcriptional level during oxidative stress [34]. Interestingly, the up-regulation of the two *CSD* genes was found to be dependent on changes in the miR398 levels. miR398 targets both the cytosolic *CSD1* and plastidic *CSD2*. Under normal growth conditions, the two closely related Cu-Zn SOD genes are transcribed but their mRNAs do not accumulate because of

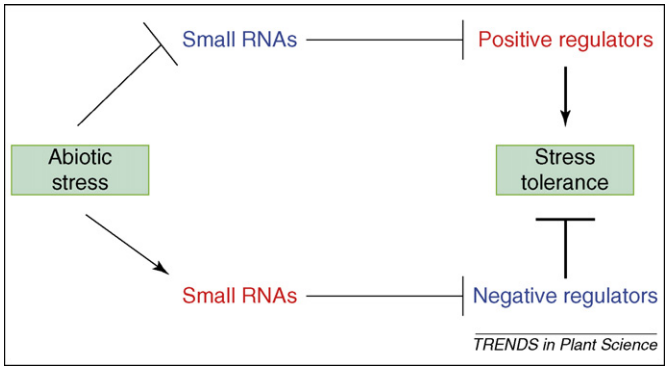

Figure 1. Two possible modes of small RNA-guided target gene regulations under abiotic stress and their impact on plant stress tolerance. Small RNAs that are positively regulated by stress might target negative regulators of stress tolerance for enhanced suppression. By contrast, small RNAs that are suppressed during stress likely target positive regulators of stress tolerance resulting in the accumulation of gene products.

Table 1. Small RNAs responsive to biotic and abiotic stress, and to nutrient deprivation in *Arabidopsis*

Small RNA	Conditions tested	Response	Validated target genes	Refs
miR398	Treated with diverse oxidative stress-causing agents such as high light levels, Cu^{2+}, Fe^{3+} and methyl viologen	Down-regulated	At1g08830 (Superoxide dismutase 1, CSD1) At2g28190 (Superoxide dismutase 2, CSD2) At3g15640 (Cytochrome *c* oxidase subunit V)	[34]
miR393	(a) Cold, dehydration, NaCl, and ABA stress (b) Leaves treated with bacterial flagellin 22 (c) Leaves infiltrated with *Pseudomonas syringae pv. tomato* (*DC3000hrcC*)	Up-regulated Up-regulated Up-regulated	At1g12820 (F-box protein, AFB3) At3g26810 (F-box protein, AFB2) Atg62980 (Auxin receptor, TIR1) At4g03190 (F-box protein, AFB1) At3g23690 (basic helix–loop–helix family protein)	[27] [33] [51]
miR395	Low sulfate levels in the media	Up-regulated	At5g10180 (Sulfate transporter, AST68) At3g22890 (ATP sulfurylase 1, APS1) At4g14680 (ATP sulfurylase 3, APS3) At5g43780 (ATP sulfurylase 4, APS4)	[28,48]
miR399	Low phosphate levels in the media	Up-regulated	At2g33770 (Ubiquitin conjugating enzyme-E2, UBC24) At3g54700 (Phosphate transporter)	[29–32]
SRO5-P5CDH natsiRNA	Salinity (NaCl) stress	Up-regulated	At5g62530 (Pyrroline-5-carboxylate dehydrogenase, P5CDH)	[74]
natsiRNAATGB2	Leaves infiltrated with *P. syringae pv. tomato* (*avrRpt2*)	Up-regulated	At4g35850 (a member of pentatricopeptide repeat containing protein family, PPRL)	[88]

miR398-guided cleavage. In response to oxidative stress, miR398 is transcriptionally down-regulated to release its suppression of *CSD1* and *CSD2* genes [34] (Table 1). Thus, the down-regulation of miR398 expression permits the accumulation of *CSD1* and *CSD2* mRNAs, which are important for plant stress resistance. Further insight into the role of miR398 in regulating the *CSD2* gene has been obtained from transgenic plants carrying miR398-resistant mutations in the *CSD2* mRNA. These plants showed much improved tolerance to diverse abiotic stress conditions compared with transgenic plants carrying the normal, miR398-susceptible *CSD2* gene [34].

Given the important role of Cu-Zn superoxide dismutases (CSDs), particularly under stress conditions, it is intriguing to ask why plants have evolved a conserved negative regulatory mechanism on the expression of two of the three CSDs involving miR398. First, there are metabolic costs associated with CSDs mRNA synthesis. Second, there is also a cost associated with miR398-guided post-transcriptional silencing. This investment suggests that miR398-guided *CSD1* and *CSD2* regulation in plant cells is unlikely to be a futile process, and this regulation could be important for multiple reasons. MicroRNA398 plays an important dual but opposite role during normal growth conditions and abiotic stress (Figure 2). Both plant development and stress resistance pathways constitute a complex network of multiple pathways. Only genes encoding proteins in the right places at the right times at optimal levels

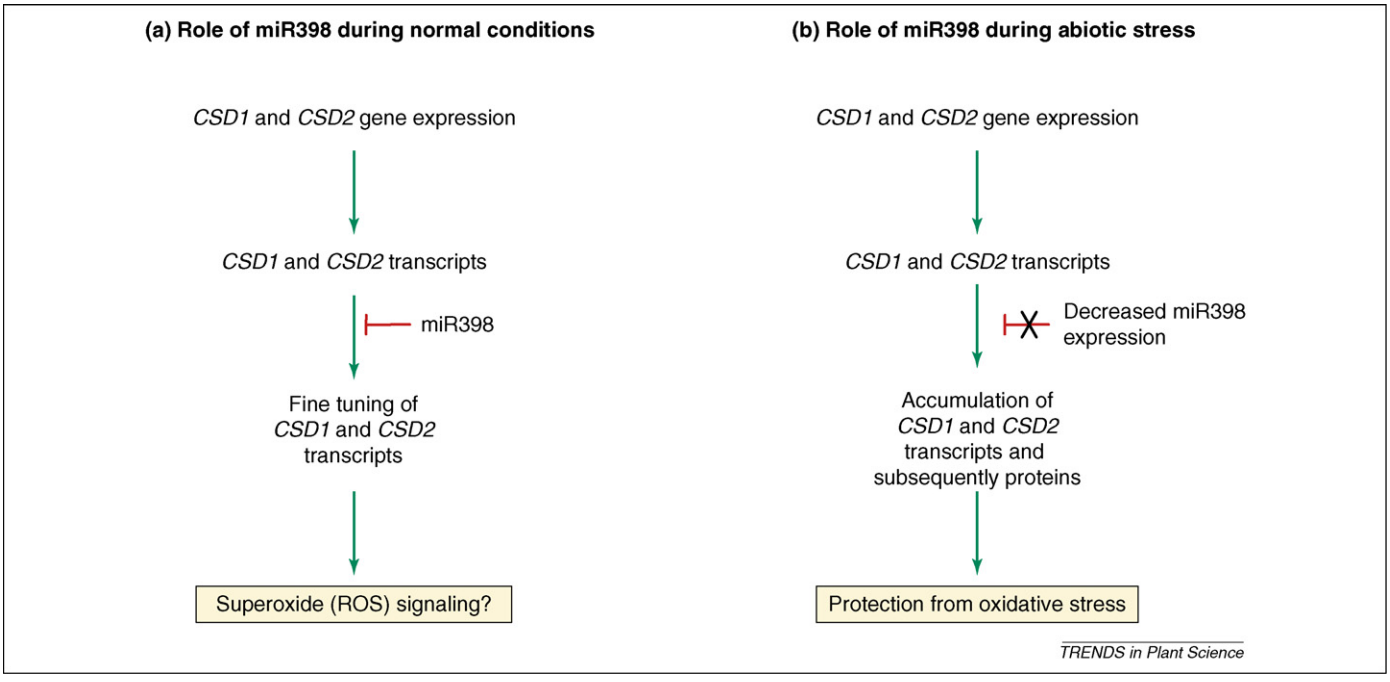

Figure 2. Model depicting a role for miR398 under **(a)** normal growth conditions and **(b)** during abiotic stress in regulating the expression of *CSD1* and *CSD2* genes at the post-transcriptional level. miR398 plays dual but opposite roles during normal growth conditions and abiotic stress. miR398 is expressed in a spatial- and temporal-specific manner under normal growth conditions and regulates the expression of *CSD1* and *CSD2* transcripts. This miR398-guided regulation might be crucial for the expression of optimal CSD1 and CSD2 that in turn regulate the levels of superoxide or other ROS required for signaling. This is still relatively unknown, and is indicated by a question mark (?). During abiotic stress, miR398 expression is suppressed and this relieves the negative regulation on two *SOD* genes resulting in the accumulation of *CSD1* and *CSD2* transcripts. The enhanced *CSD1* and *CSD2* transcripts contribute to enhanced detoxification of ROS, which can accumulate at high levels during stress.

can determine normal growth and development or stress tolerance. Therefore, protein abundance needs to be tightly coordinated in both space and time, in both processes. The abundant expression of miR398 in certain tissues under normal growth conditions suggests a role for miR398 in superoxide and H_2O_2 signaling [35] via regulation of the *CSD1* and *CSD2* genes. miR398-guided, tissue-specific post-transcriptional regulation plays a crucial role in specifying the temporal- and spatial-expression pattern of *CSD1* and *CSD2* (Figure 2a). Although the precise physiological implication for the differential accumulation of *CSD1* and *CSD2* mRNAs in different tissues is not known, it is likely that some tissues require a high level of *CSD1* and *CSD2* expression even under normal growth conditions. Knowledge about the physiological concentrations of ROS (superoxide or H_2O_2) required for signaling should shed light on the significance of miR398-guided post-transcriptional regulation under normal growth conditions. The role of miR398 during stress conditions is to promote the up-regulation of two *CSD* genes (Figure 2b). One could speculate that a change in the expression of a miRNA might be an efficient strategy for cells to coordinate changes in the expression of multiple target genes participating in the same pathway. Decreased miR398 expression during stress enables both target (*CSD1* and *CSD2*) mRNA levels to increase so that they can participate in oxidative stress management. Another gene, cytochrome *c* oxidase subunit V is also targeted by miR398 [27,28]. Further studies are required to determine whether this gene is also part of the oxidative stress network in plants.

miRNAs and nutrient homeostasis

Inorganic phosphate (P_i) is frequently a limiting factor for plant growth [39]. Plants adapt to low P_i soils in several ways: root growth and architecture are altered to access a larger soil volume; organic acids, phosphatases and nucleases are exuded to solubilize P_i or release P_i from organic sources; the capacity for P_i uptake is increased; and internal P_i is recycled [40–42]. Although some components of P_i starvation signaling in plants have been identified, the overall pathway is still poorly understood [32]. Recent findings implicate the involvement of miR399 in P_i starvation responses. The *Arabidopsis* genome encodes six MIR399 genes [27,28]. miR399 was predicted to target two genes belonging to different families: a phosphate transporter [28] and a putative ubiquitin conjugating enzyme (UBC24) possibly involved in protein degradation [27]. Both target genes function in P_i homeostasis.

miR399 has multiple target sites on the 5′UTR of the *UBC* transcript [27]. miR399 levels increase dramatically in P_i-deprived plants [29,30,32] but fall rapidly after the addition of P_i [32]. P_i starvation strongly induces the expression of all six MIR399 genes and this is rapidly reversed by P_i addition [32]. An inverse correlation between miR399 and the *UBC* mRNA levels has been observed under low P_i conditions and under P_i-sufficient conditions [29–32]. Down-regulation of UBC under low P_i conditions is important for the attenuation of primary root elongation, induction of high-affinity P_i transporters such as *AtPT1*, and acquisition of P_i to maintain P_i homeostasis [29,30]. miR399 overexpressing transgenic plants accumulated five to six times the normal P_i level in shoots and showed P_i toxicity symptoms that were phenocopied by a loss-of-function in the miR399 target gene (i.e. the *ubc* (*pho2*) mutant) [30–32,43]. *pho2* mutant plants are known to over-accumulate P_i in the shoot. Recently, *PHO2* was cloned and found to be the *UBC24* gene targeted by miR399 [31,32].

Under P_i-sufficient conditions, miR399 expression is suppressed and *UBC24* or *PHO2* is expressed and presumably participates in a ubiquitin or proteosome pathway that negatively regulates the expression of P_i transporters and controls hormonal signaling for root growth regulation to prevent the overloading of P_i. However, under limiting P_i conditions, miR399 is induced and represses *UBC24* or *PHO2* expression and, thus, alleviates the repression of P_i transporter genes and alters root growth and architecture to maximize P_i uptake. Thus, miR399 plays a key role in maintaining P_i homeostasis. The protein targets of the UBC24 or PHO2-mediated ubiquitin or proteosomal pathway are not known, but might include transcriptional activators of P_i starvation responsive genes and components of the auxin and ethylene pathways that control root growth and development under P_i stress. Interestingly, *At4*, a non-coding transcript with significant sequence complementarity to miR399 is also induced under low P_i conditions [44]. The miR399 complementary sequence in At4 homologs is highly conserved in several plant species [44]. A hairpin structure could not be predicted for the At4 precursor sequences. It is not known whether there might be an interaction between miR399 and this non-coding RNA. Recent analysis of the *At4* loss-of function mutant indicated that the At4 non-coding RNA does indeed play a role in redistributing P_i from shoots to roots under P_i deprivation [44]. It is possible that the At4 non-coding transcript can modulate the activity of miR399 by binding to the miRNA to prevent it from targeting the UBC24 or phosphate transporter, or from being degraded.

Another example of possible involvement of miRNAs in nutrient homeostasis is miR395 in sulfur starvation. For land plants, sulfur is an indispensable inorganic nutrient ranking in need next to N, P_i and K. Sulfur is taken up by roots mainly as inorganic sulfate [45]. Sulfate starvation leads to a range of physiological changes so that sulfate acquisition can be sustained to some extent and sulfate assimilation is suspended. This involves alterations in the expression levels of sulfate assimilation enzymes. MicroRNA395 is represented by six loci arranged in two clusters in *Arabidopsis* [46]. miR395 targets ATP sulfurylases (*APS1*, *APS3* and *APS4*), enzymes that catalyze the first step of the sulfur assimilation pathway [47]. miR395 also targets AST68, a low-affinity sulfate transporter (At5g10180, AtSULTR2;1) [48] (Table 1). AST68 is implicated in the internal translocation of sulfate from roots to shoots [49,50]. Thus, miR395 potentially plays an important role in coordinating changes in sulfate translocation and assimilation. miR395 was induced under conditions of low sulfate, whereas *APS1* transcript levels decreased [28]. By contrast, *APS1* transcripts were abundantly expressed when miR395 expression could not be detected under sulfur-sufficient conditions [28]. The functional significance of sulfate regulation of miR395 and its targets

AST68, *APS1*, *APS3* and *APS4* remain to be investigated. Nevertheless, the available evidence implies an important role for miR395 in regulating sulfate homeostasis.

Other abiotic stress-regulated small RNAs and their target genes

High-through-put gene expression analysis of plants under abiotic stress indicated that several hundred genes have modulated expression [19–23]. Some of these genes are up-regulated or induced, and others are down-regulated under stress conditions. The up- or down-regulation of genes appears to be dependent on their roles. MicroRNAs that are up-regulated by stress might down-regulate their target genes, which might be negative regulators of stress tolerance (e.g. repressors of stress-responsive genes and genes involved in plant processes that are inhibited by stresses e.g. cell division and expansion); downregulation of miRNAs under stress might result in accumulation of their target gene mRNAs, which might positively regulate stress tolerance (Figure 1). Sequence analysis of a stress-treated *Arabidopsis* small RNA library indicated that miR393 was the most abundantly expressed miRNA based on the number of times it appeared [27]. miR393 was also found to be induced in response to flagellin [33] or *Pseudomonas syringae* treatment [51] (Table 1). Stress-specific regulation of miRNAs was also observed, for example, miR319c is up-regulated by cold but not by dehydration, salt or ABA [27]. Microarray data from Genevestigator (https://www.genevestigator.ethz.ch) [52] suggest that several miRNA target genes are altered under diverse stress conditions, although this information needs further scrutiny because of differences in the growth conditions, duration of the treatment and age of the plants used.

The predicted target genes of miR393 include four putative ubiquitin E3 ligase SCF complex F-box proteins and a basic-helix–loop–helix family protein [27,28]. One of the F-box family proteins (At4g03190) is identical to Glucose Repression Resistance-like protein 1 (GRR1). Ubiquitin E3 ligases confer substrate specificity to the ubiquitin or 26S proteosome pathway, which mediate regulated protein degradation and, thus, stress response and developmental processes. Protein degradation serves at least two physiological functions in plant stress responses (i.e. gene regulation and senescence). Most of the abiotic stresses induce senescence. Senescence of older leaves helps not only to supply the nutrients (mainly N) to young leaves and reproductive parts, but also minimizes water loss under drought stress. Furthermore, ubiquitination also regulates gene expression under stress. During cold acclimation, Inducer of CBF expression 1 (ICE1) protein is activated, inducing the expression of C-repeat (CRT)-binding factors (CBFs) and other transcription factors [53,54]. The HOS1 (high expression of osmotically responsive genes 1) protein, a ubiquitin E3 ligase, negatively regulates cold responses through the ubiquitination of ICE1 [55]. The miR393 target gene, At3G26810, a putative ubiquitin E3 ligase, is downregulated by 0.92, 0.66, 0.55 and 0.66-fold under drought, salt, cold and ABA, respectively, compared with non-stress conditions (Genevestigator response viewer data). Furthermore, other miR393 target genes coding for E3 ubiquitin ligases At1g12820 and At3g62980 (an auxin receptor, transport inhibitor response 1) showed downregulation under cold stress (Genevestigator response viewer https://www.genevestigator.ethz.ch). The Response Viewer tool provides gene expression data for control and stress conditions, that is, biotic and abiotic stresses, hormones and chemicals from several representative experiments [52]. Studies of miR393 regulation of these E3 ligases and their role in abiotic stress responses should provide insights into the mechanism of regulated proteolysis-mediated abiotic stress tolerance.

Energy and carbon requirements for growth and development are provided by sugars, the primary product of photosynthesis. Hence, many metabolic processes are regulated by sugar concentration, which is in turn influenced by abiotic stresses. The plant stress hormone, abscisic acid (ABA) plays a crucial role in cellular sugar budget-mediated regulation of plant growth and development [56]. One of the miR393 target genes, At4g03190, encodes an F-box protein that shows similarity to GRR1, a yeast protein involved in glucose repression. Genevestigator response viewer data [52] suggest that At4g03190 is down-regulated in response to cold and ABA. Investigations into the role of miR393 and its target At4g03190 might shed light on sugar sensing in plants under stress.

The levels of several poplar miRNAs (miR156, miR162, mi164, mi475, mi480 and mi481) declined under mechanical stress conditions [57]. Interestingly, some of these miRNAs are poplar-specific and are induced under stress conditions whereas some other miRNAs such as miR156, miR162 and miR164 are associated with development. The functional roles of these putative mechanical stress-responsive miRNAs remain to be determined experimentally.

miRNAs and growth and development under abiotic stresses

Crop yield under abiotic stresses depends not only on the mere survival of plants under stress conditions but also on the phenological and developmental plasticity of plants. Under abiotic stress conditions, plants adjust the durations of phenological phases, and the rate of developmental processes, which modify biomass and harvest index. Changes in the duration of various phenological phases (e.g. vegetative phase, days to flowering and grain development duration) help plants to avoid critical growth phases under stress conditions. Tolerant genotypes often enhance their growth rate to compensate for the reductions in phenological durations. Reproductive development appears to be the phase of crop development that is the most susceptible to abiotic stresses given that any damage at this stage is irrecoverable. Drought stress reduces days to flowering in wheat [58] but delays flowering in rice [59]. In maize, drought stress increases anthesis to silking interval [60]. Reduction in reproductive organ number and size helps plants to use the available resources efficiently so that some viable healthy seeds are produced. However, to date the molecular basis of phenological and developmental plasticity under abiotic stress is poorly understood. Interestingly, our analysis of microarray data from Genevestigator [52] suggests that many of the miRNA target genes involved in growth and development are stress-regulated as well. Extensive molecular, physiological and even anatomical changes take place in plants in response to a stressful

environment such that plants under a specific stress condition might be viewed as entering a particular developmental phase. MicroRNA160, miR164 and their target genes are involved in the regulation of root growth. Roots play a pivotal role in the acquisition and transport of water and nutrients, and root-based hormonal signal (ABA) is an important determinant of stomatal responses.

Under conditions of drought, roots can adapt to continue growth to acquire water and nutrients from deep soil layers. Overexpression of miR160, which targets auxin response factors (ARFs) resulted in agravitrophic roots and increased the number of lateral roots, whereas overexpression of miR160-resistant ARF16 resulted in reduced lateral roots and reduced fertility [61]. By contrast, transgenic *Arabidopsis* overexpressing miR164, which targets NAC transcription factors, exhibited reduced lateral roots, whereas overexpression of miR164-resistant NAC1 resulted in increased number of lateral roots [62].

Leaf development is also regulated by miRNAs. Leaf development determines the source size (photosynthetic area) and area for transpiration and, hence, regulation of leaf development is crucial for abiotic stress tolerance. Plants overexpressing miR159-resistant MYB33 exhibited reductions in size, petiole length, apical dominance and fertility, and had round leaves [63]. Transgenic *Arabidopsis* overexpressing miR160-resistant ARF17 had extra cotyledons, leaf defects, extra petals and reduced fertility [64]. Similarly, overexpression of miR164-resistant CUC1, miR164-resistant CUC2, miR165 or miR166-resistant PHB and miR165 or 166-resistant REV in transgenic *Arabidopsis* resulted in leaf polarity defects [65–67].

The reproductive phase is also sensitive to abiotic stresses. Plants modulate flowering time and flower number (sink size) in response to abiotic stresses. Overexpression of some miRNAs results in alterations of flowering time. Overexpression of miR156, which targets the SPL family of transcription factors, showed enhanced leaf initiation, decreased apical dominance and delayed flowering time [68]. Similarly transgenic *Arabidopsis* overexpressing gibberellin-regulated miR159 and miR319 showed a delay in flowering time [69,70]. By contrast, overexpression of miR172, which targets AP2-type transcription factors, resulted in early flowering [71,72]. In addition to the time of flowering, fertility is highly affected by abiotic stresses. Transgenic overexpression of miR159 and miR166 resulted in enhanced male sterility and female sterility, respectively, under non-stress conditions [70,73].

siRNAs and stress responses

Endogenous siRNAs are synthesized from long double-stranded RNAs (dsRNAs). The endogenous sources of dsRNAs are: (i) miRNA-directed cleavage products of non-coding transcripts, which are then converted into dsRNAs by RNA-dependent RNA polymerases (RDRs); (ii) dsRNAs formed from the mRNAs encoded by natural *cis*-antisense gene pairs [74]; and (iii) dsRNAs generated from heterochromatin and DNA repeats [8]. The siRNAs produced by miRNA-directed cleavage of mRNAs are referred to as *trans*-acting siRNAs (ta-siRNAs); the siRNAs derived from dsRNAs formed from the mRNAs encoded by natural *cis*-antisense gene pairs are called natural antisense transcript-derived siRNAs (nat-siRNAs). RDRs and DCL-like proteins process the dsRNAs formed from different sources. The biogenesis of different classes of siRNAs is carried out by specific RDR–DCL combinations [7,8].

ta-siRNAs biogenesis begins with miRNA-directed cleavage of target mRNAs and these cleaved single-stranded RNAs are recognized by SUPPRESSOR OF GENE SILENCING 3 (SGS3, At5g23570), a coiled-coil protein with a zinc finger domain, followed by synthesis of the complementary RNA strand by RDR6 (At3g49500 = SUPPRESSOR OF GENE SILENCING 2, SILENCING DEFECTIVE 1). These dsRNAs are then cleaved by DCL4 to produce 21 nt ta-siRNAs [75–77].

Genome analyses have revealed thousands of genes in convergent overlapping pairs that can generate complementary transcripts [78–80]. In addition, various expression profiling approaches showed widespread antisense transcription throughout genomes in plants [81,82]. From overlapping genes on opposite strands of DNA, *cis*-natural antisense transcripts (NATs; endogenous coding or non-protein-coding RNAs with sequence complementarity to other transcripts) are generated [74]. The biogenesis of nat-siRNAs begins with the formation of dsRNAs by annealing sense and antisense transcripts. These dsRNAs are processed by DCL2, RDR6, SGS3 and a plant-specific RNA polymerase, NRPD1A, to generate a 24-nt nat-siRNA, which then directs the biogenesis of 21-nt nat-siRNAs by DCL1 [74]. The third type of siRNAs (24 nt) is generated by DCL3, RDR2 and NRPD1A by processing RNAs from transposons, 5S rRNA genes and other repeats [8,83].

Work on the founding member of nat-siRNAs, which is derived from a *cis*-NAT gene pair of SRO5 and P5CDH genes, demonstrated an important role of nat-siRNAs in osmoprotection and oxidative stress management under salt stress in *Arabidopsis* [74] (Table 1). Salt stress-induced SRO5 mRNA complements the P5CDH mRNA to produce a dsRNA, which is processed by a siRNA biogenesis pathway requiring DCL2, RDR6, SGS3 and NRPD1A to produce a 24-nt nat-siRNA. The 24-nt nat-siRNA guides the cleavage of the P5CDH transcript to further produce 21-nt nat-siRNAs by DCL1. These nat-siRNAs all guide cleavage of P5CDH mRNAs, suppressing proline degradation and, thus, allowing proline accumulation. Downregulation of P5CDH also causes P5C-mediated ROS accumulation. The SRO5 protein is targeted to mitochondria, the site of proline catabolism [74]. SRO5 is similar to RADICAL INDUCED CELL DEATH 1 (RCD1), which prevents ROS-induced cell death, given that *rcd1* plants are hypersensitive to ROS-induced cell death [84]. High salt stress causes accumulation of H_2O_2, and both salt and H_2O_2 induce the expression of SRO5. *sro5* mutant plants also showed hypersensitivity to ROS (H_2O_2). These findings suggest that ROS detoxification under salt stress is mediated by the SRO5 protein [74]. Thus, the SRO5-P5CDH nat-siRNAs together with the P5CDH and SRO5 proteins form an important regulatory loop controlling proline and ROS production and stress tolerance [74].

Conclusions and outlook

Extensive efforts over the past two decades have identified thousands of stress-regulated genes. With the recent identification of miRNAs and siRNAs as components of stress response, another level of gene regulation has been revealed. The evidence thus far suggests important roles for these small RNAs in stress response. The extent of small RNA involvement in abiotic stress response should become clear in the next several years if sufficient effort can be directed to these studies in *Arabidopsis* as well as in crop plants.

Most miRNAs show dynamic expression patterns. Some miRNAs are expressed only during certain developmental stages or tissues, whereas others are responsive to hormones, nutrient deprivation or other abiotic stresses [11,27,28,34,85]. These observations imply that miRNA expression is controlled at the level of transcription. However, it is likely that miRNA expression can also be controlled at the level of processing and stability, like other RNAs. An important question is how is miRNA expression controlled at the transcriptional level in response to environmental or developmental changes? Connecting miRNAs to both upstream and downstream events will place them within the regulatory networks that govern diverse physiological processes. A recent study indicated that PHOSPHATE STARVATION RESPONSE 1 (PHR1), a MYB-like transcription factor, is one of the factors responsible for miR399 induction in response to P_i deprivation because the induction of miR399 is decreased but not completely blocked in the *phr1* mutant under low phosphate conditions [32]. Future studies aimed at dissecting the promoter elements of the miRNA genes should shed light on transcriptional regulation of miRNA genes.

NAT-siRNAs have recently emerged as important players in plant stress responses. A pair of natural antisense transcript (NAT) genes can give rise to both 24 nt and 21 nt siRNAs in response to salt stress and are important for salt tolerance in *Arabidopsis* [74]. A nat-siRNA that is specifically induced by the bacterial pathogen *Pseudomonas syringae* carrying the avirulence gene *avrRpt2* plays an important role in RPS2-mediated race-specific disease resistance [86] (Table 1). Recent genome-wide analyses have revealed the widespread existence of NATs in eukaryotic genomes [78–80]. NATs might have the potential to generate nat-siRNAs for gene regulation under various stress conditions. This hypothesis is well supported by the presence of several hundred potential nat-siRNAs in small RNA databases, including the MPSS [87] and ASRP databases [88]. The current ASRP and MPSS datasets were generated from untreated plants only [89]. We believe that many more nat-siRNAs will be discovered in the future by using stress-treated samples. Further studies of stress-regulated miRNAs and siRNAs and their target genes in plants should identify new components in plant stress resistance pathways and help to elucidate the complex regulatory network underlying plant stress responses.

Many genes have been shown to confer marginal improvements in stress tolerance when overexpressed in transgenic plants [22]. A combination of previously reported as well as novel approaches will be needed to increase plant abiotic and biotic stress resistance to levels high enough for field application in crops. Manipulation of small RNA-guided gene regulation represents a novel and feasible approach to improve plant stress tolerance [34].

In summary, an understanding of post-transcriptional gene regulation by small RNAs under abiotic stress is crucial for understanding and improving stress tolerance in crop plants. *In silico* identification of miR395, miR398 and miR399 homologs in about two dozen diverse plant species suggests that these miRNAs are conserved across species boundaries. Conservation of these miRNAs implies that they have conserved biological functions. Appropriate manipulation of miRNA target genes should help overcome post-transcriptional gene silencing and, thus, might lead to better expression of engineered traits in transgenic plants.

Acknowledgements

The work in R.S.'s laboratory is supported by the Oklahoma Agricultural Experiment Station and OCAST, and the work in J-K.Z.'s laboratory is supported by the National Institutes of Health grants R01GM0707501 and R01GM59138.

References

1 Dreyfuss, G. *et al.* (2002) Messenger RNA binding proteins and the messages they carry. *Nat. Rev. Mol. Cell Biol.* 3, 195–205
2 Gebauer, F. and Hentze, M.W. (2004) Molecular mechanisms of translational control. *Nat. Rev. Mol. Cell Biol.* 5, 827–835
3 Lopez de Heredia, M. and Jansen, R.P. (2004) mRNA localization and the cytoskeleton. *Curr. Opin. Cell Biol.* 16, 80–85
4 Ambros, V. (2004) The functions of animal microRNAs. *Nature* 431, 350–355
5 Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281–297
6 Baulcombe, D. (2004) RNA silencing in plants. *Nature* 431, 356–363
7 Jones-Rhoades, M.W. *et al.* (2006) MicroRNAs and their regulatory roles in plants. *Annu. Rev. Plant Biol.* 57, 19–53
8 Mallory, A.C. and Vaucheret, H. (2006) Functions of microRNAs and related small RNAs in plants. *Nat. Genet.* 38, S31–S36
9 Lu, C. and Federoff, N. (2000) A mutation in the *Arabidopsis HYL1* gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin, and cytokinin. *Plant Cell* 12, 2351–2366
10 McConnell, J.R. *et al.* (2001) Role of PHABULOSA and PHAVULOTA in determining radial patterning in shoots. *Nature* 411, 709–713
11 Park, W. *et al.* (2002) CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr. Biol.* 12, 1484–1495
12 Schauer, S.E. *et al.* (2002) DICER-LIKE1: blind men and elephants in *Arabidopsis* development. *Trends Plant Sci.* 7, 487–491
13 Vaucheret, H. *et al.* (2004) The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. *Genes Dev.* 18, 1187–1197
14 Lobbes, D. *et al.* (2006) SERRATE: a new player on the plant microRNA scene. *EMBO Rep.* 7, 1052–1058
15 Yang, L. *et al.* (2006) SERRATE is a novel nuclear regulator in primary microRNA processing in *Arabidopsis*. *Plant J.* 47, 841–850
16 Carrington, J.C. and Ambros, V. (2003) Role of microRNAs in plant and animal development. *Science* 301, 336–339
17 Dugas, D.V. and Bartel, B. (2004) MicroRNA regulation of gene expression in plants. *Curr. Opin. Plant Biol.* 7, 512–520
18 Kidner, C.A. and Martienssen, R.A. (2005) The developmental role of microRNA in plants. *Curr. Opin. Plant Biol.* 8, 38–44
19 Fowler, S. and Thomashow, M.F. (2002) *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* 14, 1675–1690
20 Zhu, J-K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53, 247–273
21 Seki, M. *et al.* (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *Plant J.* 31, 279–292

22 Bartels, D. and Sunkar, R. (2005) Drought and salt tolerance in plants. *Crit. Rev. Plant Sci.* 24, 23–58
23 Shinozaki, K. and Yamaguchi-Shinozaki, K. (2007) Gene networks involved in drought stress response and tolerance. *J. Exp. Bot.* 58, 221–227
24 Kawaguchi, R. and Bailey-Serres, J. (2002) Regulation of translational initiation in plants. *Curr. Opin. Plant Biol.* 5, 460–465
25 Kawaguchi, R. *et al.* (2003) Water-deficit-induced translational control in *Nicotiana tabacum*. *Plant Cell Environ.* 26, 221–229
26 Kawaguchi, R. *et al.* (2004) Differential mRNA translation contributes to gene regulation under non-stress and dehydration stress conditions in *Arabidopsis thaliana*. *Plant J.* 38, 823–839
27 Sunkar, R. and Zhu, J-K. (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. *Plant Cell* 16, 2001–2019
28 Jones-Rhoades, M.W. and Bartel, D.P. (2004) Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. *Mol. Cell* 14, 787–799
29 Fujii, H. *et al.* (2005) A miRNA involved in phosphate-starvation response in *Arabidopsis*. *Curr. Biol.* 15, 2038–2043
30 Chiou, T.J. *et al.* (2006) Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. *Plant Cell* 18, 412–421
31 Aung, K. *et al.* (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol.* 141, 1000–1011
32 Bari, R. *et al.* (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol.* 141, 988–999
33 Navarro, L. *et al.* (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science* 312, 436–439
34 Sunkar, R. *et al.* (2006) Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. *Plant Cell* 18, 2051–2065
35 Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55, 373–399
36 Mittler, R. *et al.* (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.* 9, 490–498
37 Bowler, C. *et al.* (1991) Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J.* 10, 1723–1732
38 Kliebenstein, D.J. *et al.* (1998) Superoxide dismutase in *Arabidopsis*: an eclectic enzyme family with disparate regulation and protein localization. *Plant Physiol.* 118, 637–650
39 Marschner, H. (1995) *Mineral Nutrition of Higher Plants* (2nd edn), Academic Press
40 Raghothama, K.G. (1999) Phosphate acquisition. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 665–693
41 Abel, S. *et al.* (2002) Phosphate sensing in higher plants. *Physiol. Plant.* 115, 1–8
42 Poirier, Y. and Bucher, M. (2002) Phosphate transport and homeostasis in *Arabidopsis*. In *Arabidopsis* (Somerville, C.R. and Meyerowitz, E.M., eds), American Society of Plant Biologists, DOI: 10.1199/tab.0024 (http://www.aspb.org/publications/*Arabidopsis*)
43 Delhaize, E. and Randall, P.J. (1995) Characterization of a phosphate accumulator mutant of *Arabidopsis thaliana*. *Plant Physiol.* 107, 207–213
44 Shin, H. *et al.* (2006) Loss of At4 function impacts phosphate distribution between the roots and the shoots during phosphate starvation. *Plant J.* 45, 712–726
45 Nikiforova, V.J. *et al.* (2006) Effect of sulfur availability on the integrity of amino acid biosynthesis in plants. *Amino Acids* 30, 173–183
46 Guddeti, S. *et al.* (2005) Molecular evolution of the rice miR395 gene family. *Cell Res.* 15, 631–638
47 Lappartient, A.G. *et al.* (1999) Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. *Plant J.* 18, 89–95
48 Allen, E. *et al.* (2005) microRNA-directed phasing during *trans*-acting siRNA biogenesis in plants. *Cell* 121, 207–221
49 Takahashi, H. *et al.* (1997) Regulation of sulfur assimilation in higher plants: a sulfate transporter induced in sulfate-starved roots plays a central role in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A.* 94, 11102–11107
50 Takahashi, H. *et al.* (2000) The roles of three functional sulphate transporters involved in uptake and translocation of sulphate in *Arabidopsis thaliana*. *Plant J.* 23, 171–182
51 Fahlgren, N. *et al.* (2007) High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of MIRNA genes. *PLoS ONE* 2, e219
52 Zimmermann, P. *et al.* (2004) GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. *Plant Physiol.* 136, 2621–2632
53 Chinnusamy, V. *et al.* (2003) ICE1, a regulator of cold induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes Dev.* 17, 1043–1054
54 Chinnusamy, V. *et al.* (2006) Gene regulation during cold acclimation in plants. *Physiol. Plant.* 126, 52–61
55 Dong, C.H. *et al.* (2006) The negative regulator of plant cold responses, HOS1, is a RING E3 ligase that mediates the ubiquitination and degradation of ICE1. *Proc. Natl. Acad. Sci. U. S. A.* 103, 8281–8286
56 Rolland, F. *et al.* (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.* 57, 675–709
57 Lu, S. *et al.* (2005) Novel and mechanical stress-responsive microRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. *Plant Cell* 17, 2186–2203
58 Kato, K. and Yokoyama, H. (1992) Geographical variation in heading characters among wheat landraces, *Triticum aestivum* L., and its implication for their adaptability. *Theor. Appl. Genet.* 84, 259–265
59 Boonjung, H. and Fukai, S. (1996) Effect of soil water deficit at different growth stages on rice growth and yield under upland conditions. 2. Phenology, biomass production and yield. *Field Crop. Res.* 48, 47–55
60 Ribaut, J.M. *et al.* (1996) Identification of quantitative trait loci under drought conditions in tropical maize: 1 flowering parameters and the anthesis-silking interval. *Theor. Appl. Genet.* 92, 905–914
61 Wang, J.W. *et al.* (2005) Control of root cap formation by microRNA-targeted auxin response factors in *Arabidopsis*. *Plant Cell* 17, 2204–2216
62 Guo, H.S. *et al.* (2005) MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for *Arabidopsis* lateral root development. *Plant Cell* 17, 1376–1387
63 Millar, A.A. and Gubler, F. (2005) The *Arabidopsis* GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. *Plant Cell* 17, 705–721
64 Mallory, A.C. *et al.* (2005) MicroRNA-directed regulation of *Arabidopsis* AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. *Plant Cell* 17, 1360–1375
65 Laufs, P. *et al.* (2004) MicroRNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. *Development* 131, 4311–4322
66 Mallory, A.C. *et al.* (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Curr. Biol.* 14, 1035–1046
67 Mallory, A.C. *et al.* (2004) MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5′ region. *EMBO J.* 23, 3356–3364
68 Schwab, R. *et al.* (2005) Specific effects of microRNAs on the plant transcriptome. *Dev. Cell* 8, 517–527
69 Palatnik, J.F. *et al.* (2003) Control of leaf morphogenesis by microRNAs. *Nature* 425, 257–263
70 Achard, P. *et al.* (2004) Modulation of floral development by a gibberellin-regulated microRNA. *Development* 131, 3357–3365
71 Chen, X. (2004) A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. *Science* 303, 2022–2025
72 Aukerman, M.J. and Sakai, H. (2003) Regulation of flowering time and floral organ identity by a microRNA and its APETALA2-like target genes. *Plant Cell* 15, 2730–2741
73 Williams, L. *et al.* (2005) Regulation of *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHDZIP target genes. *Development* 132, 3657–3668
74 Borsani, O. *et al.* (2005) Endogenous siRNAs derived from a pair of natural *cis*-antisense transcripts regulate salt tolerance in *Arabidopsis*. *Cell* 123, 1279–1291
75 Xie, Z. *et al.* (2005) DICER-LIKE 4 functions *in trans*-acting small interfering RNA biogenesis and vegetative phase change in

Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U. S. A.* 102, 12984–12989
76 Yoshikawa, M. *et al.* (2005) A pathway for the biogenesis of *trans*-acting siRNAs in *Arabidopsis*. *Genes Dev.* 19, 2164–2175
77 Ronemus, M. *et al.* (2006) MicroRNA-targeted and small interfering RNA-mediated mRNA degradation is regulated by argonaute, Dicer, and RNA-dependent RNA polymerase in *Arabidopsis*. *Plant Cell* 18, 1559–1574
78 Boi, S. *et al.* (2004) Shedding light on the dark side of the genome: overlapping genes in higher eukaryotes. *Curr. Genomics* 5, 509–524
79 Wang, X.J. *et al.* (2005) Genome-wide prediction and identification of *cis*-natural antisense transcripts in *Arabidopsis thaliana*. *Genome Biol.* 6, R30
80 Wang, H. *et al.* (2006) Prediction of *trans*-antisense transcripts in *Arabidopsis thaliana*. *Genome Biol.* 7, R92
81 Brenner, S. *et al.* (2000) Gene expression analysis by massively parallel signature sequencing (MPSS) on microbead arrays. *Nat. Biotechnol.* 18, 630–634
82 Yamada, K. *et al.* (2003) Empirical analysis of transcriptional activity in the *Arabidopsis* genome. *Science* 302, 842–846
83 Chan, S.W. *et al.* (2004) RNA silencing genes control *de novo* DNA methylation. *Science* 303, 1336
84 Ahlfors, R. *et al.* (2004) *Arabidopsis* RADICAL-INDUCED CELL DEATH1 belongs to the WWE protein–protein interaction domain protein family and modulates abscisic acid, ethylene, and methyl jasmonate responses. *Plant Cell* 16, 1925–1937
85 Sunkar, R. *et al.* (2005) Cloning and characterization of microRNAs from rice. *Plant Cell* 17, 1397–1411
86 Katiyar-Agarwal, S. *et al.* (2006) A pathogen-inducible endogenous siRNA in plant immunity. *Proc. Natl. Acad. Sci. U. S. A.* 103, 18002–18007
87 Nakano, M. *et al.* (2006) Plant MPSS databases: signature-based transcriptional resources for analyses of mRNA and small RNA. *Nucleic Acids Res.* 34, D731–D735
88 Gustafson, A.M. *et al.* (2005) ASRP: the *Arabidopsis* small RNA project database. *Nucleic Acids Res.* 33, D637–D640
89 Lu, C. *et al.* (2005) Elucidation of the small RNA component of the transcriptome. *Science* 309, 1567–1569
90 Lee, R.C. *et al.* (1993) The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 75, 843–854
91 Wightman, B. *et al.* (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* 75, 855–862