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5

Facing the Environment: Small RNAs and the Regulation of Gene Expression Under Abiotic Stress in Plants

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1. Introduction

Plant growth and development is highly dependent on a variety of environmental conditions such as temperature, light, water availability and soil conditions that strongly affect productivity worldwide. Over the past years, several reports have raised awareness for the fact that extreme weather conditions are predicted to become more frequent in a near future, which is likely to have a strong impact in crop production. For instance, it is estimated that by 2030, global water demand solely for agriculture may have increased by more than 30% as a consequence of foreseen climate changes (Foresight, 2011). Therefore, there is an urgency to understand how plants behave when facing adverse conditions, in order to reduce productivity losses in sub-optimal environments.

Plants have developed different strategies to cope with abiotic stress conditions. Upon environmental stimuli, changes in metabolism, signal transduction pathways and gene expression can be detected (Shinozaki & Yamaguchi-Shinozaki, 2007). Post-transcriptional regulatory mechanisms, as well as epigenetic and post-translational modifications, like ubiquitination and sumoylation, seem to play an important role in the regulation of gene expression in stress conditions (reviewed in Hirayama & Shinozaki, 2010).

In this chapter we will provide evidences of the involvement of small RNAs (sRNAs) in the regulation of gene expression as response to abiotic stress. The role of microRNAs (miRNAs) and other sRNAs under water deficit, high salinity, cold and oxidative stress, among others, as well as their relation to hormone signalling in plants, will be reviewed. Moreover, we will show that key enzymes involved in sRNA synthesis pathways also seem to be regulated upon environmental stimuli, affecting the expression of most sRNAs and consequently of several genes.

2. Plant small RNAs

Evidence for the existence of RNA-mediated silencing mechanisms in plants first appeared in the late 1990's, when short antisense RNA molecules were isolated from tomato plants where post-transcriptional gene silencing (PTGS) had been detected (Hamilton & Baulcombe, 1999). Since then, the knowledge on sRNAs has broadened and these molecules have been identified as important players in a wide variety of processes in plants. Back in 2002, when the first set of plant microRNAs (miRNAs) was cloned (Reinhart et al., 2002), there were 218 entries in the public miRNA database (miRBase; Griffiths-Jones, 2004) whereas nowadays more than 15 000 entries can be found.

The biosynthesis of sRNAs is triggered by the presence of double-stranded RNA (dsRNA) molecules that are processed into small RNA duplexes by RNase III Dicer-like (DCL) proteins (reviewed by Bartel, 2004). S-adenosyl methionine-dependent methyltransferase Hua Enhancer 1 (HEN1) methylates these molecules at the 3' end, in a sequence-independent manner, protecting them from uridylation and degradation (Park et al., 2002; Li et al., 2005; Yu et al., 2005). There are 4 loci in arabidopsis and rice (*Oryza sativa*) that encode for DCL proteins (Liu et al., 2005). Although some functional redundancy has been observed, each DCL seems to have a specific role in sRNA biosynthesis pathways (Bouche et al., 2006; Henderson et al., 2006).

Following methylation, sRNAs are incorporated into ARGONAUTE (AGO) proteins, in order to act upon targets in a sequence-specific manner (Llave et al., 2002b; Rhoades et al., 2002; Vazquez et al., 2004). Plants have multiple loci encoding for AGO proteins: 10 are known in arabidopsis and at least 18 in rice (Mallory & Vaucheret, 2010). AGO4, AGO6 and AGO9 are usually involved in silencing at the transcriptional level, by association with 24-nt sRNAs, whereas AGO1 and AGO7 participate in post-transcriptional gene silencing and are usually loaded with 21 and 22-nt sRNAs (reviewed in Mallory & Vaucheret, 2010). Recently, a role for AGO2 in responses to virus infection has also been described (Harvey et al., 2011). With few exceptions, the main characteristics that influence the loading of AGO proteins in plants are both the size of the sRNAs and the nature of the nucleotide at the 5' end (Mi et al., 2008; Mallory & Vaucheret, 2010).

Depending mainly on their origin, sRNAs can be roughly divided into two distinct classes: miRNAs (Fig.1A) and small interfering RNAs (siRNAs, Fig.1B and 1C) (Hamilton et al., 2002; Rajagopalan et al., 2006).

2.1 MicroRNAs

miRNAs are usually transcribed from intergenic regions (Llave et al., 2002a; Reinhart et al., 2002) by Polymerase II (Pol II) into 5' capped and 3' polyadenylated primary transcripts, in a way similar to protein-coding genes (Xie et al., 2005). These precursors (named pri-miRNAs) have regions of self-complementarity and can therefore fold back into stem-loop structures (Park et al., 2002; Meyers et al., 2008), yielding the dsRNA region, needed to trigger RNA silencing mechanisms. The hairpin precursors, called pre-miRNAs, are released from the pri-miRNAs by DCL1 in the nucleus. DCL1 then processes the pre-miRNAs into smaller duplexes bearing the mature miRNA and the complementary strand (miRNA*) (Reinhart et al., 2002; Henderson et al., 2006). In plants, both HYPONASTIC LEAVES1 (HYL1) and SERRATE (SE) proteins seem to interact with DCL1 and are also required for this process (Han et al., 2004; Lobbes et al., 2006).

At this point in the biosynthesis pathway, miRNAs can either stay in the nucleus or be transported to the cytoplasm, a process that seems to be dependent on HASTY protein, an exportin homolog (Bartel, 2004; Park et al., 2005). Either way, mature miRNAs are loaded into AGO proteins, and act upon highly or perfectly complementary targets by promoting their cleavage or repressing translation (Llave et al., 2002b; Rhoades et al., 2002; Chen, 2004).
Most miRNAs are 21-nt long and are generally loaded into AGO1 that has both a small RNA-binding PAZ domain and a catalytic PIWI domain, and mediates miRNA-guided cleavage of complementary target transcripts (Vaucheret et al., 2004; Baumberger & Baulcombe, 2005). Opposite to what was known for animals, in 2005 Baumberger and Baulcombe reported that immunoprecipitation assays suggested that AGO1 alone could be enough for RNA induced silencing mechanisms in plants. Recently, however, Hsp90, a chaperone involved in protein folding, was co-purified with AGO1 (Iki et al., 2010), suggesting these two proteins could be part of a plant RNA silencing complex. In the same work, Hsp90 was found to be required for the loading of sRNAs into AGO1, presumably by inducing conformational changes in this protein (Iki et al., 2010).
There are also other miRNAs that are processed into 22-nt molecules, a process that is thought to be related to asymmetry in the hairpin precursor that is cleaved into a 22-nt mature miRNA and a 21-nt miRNA* (Cuperus et al., 2010). Recently, these molecules that are also loaded into AGO1 were found to trigger the production of secondary sRNAs from the 3' product of miRNA-directed cleavage (Chen et al., 2010; Cuperus et al., 2010).

2.2 Small interfering RNAs

A second and less studied group of sRNAs is known to exist in plants. These molecules, generally called siRNAs, are cleaved by DCL proteins from long dsRNA molecules that do not form hairpins (Carrington & Ambros, 2003). These are produced by either the transcription of inverted repeats or by the action of RNA-dependent RNA polymerases (RDRs) that convert single-stranded RNA transcripts into dsRNA (Dalmay et al., 2000).
The best studied siRNAs in plants fall into two distinct categories: RDR2-dependent (or heterochromatin associated) siRNAs (Fig.1B) and *trans*-acting siRNAs (ta-siRNAs, Fig.1C) (Ramachandran & Chen, 2008).
The first sub-group comprises siRNAs that are transcribed by RNA polymerase IV (Pol IV) and further processed to dsRNA by RDR2 (Herr et al., 2005). These sRNAs are mostly cleaved into 24-nt duplexes by DCL3 (Gasciolli et al., 2005) and loaded into AGO4 to guide target silencing, usually by promoting DNA or histone methylation (Zilberman et al., 2003; Herr et al., 2005).
The other sub-group, gathers the small RNAs that act upon transcripts that are very different from the genes that originated them (Vazquez et al., 2004). The biogenesis of these ta-siRNAs requires miRNA-AGO1-mediated cleavage of target transcripts, called *TAS* genes (Allen et al., 2005; Allen & Howell, 2010), and seems to be related to the production of 22-nt miRNAs (Chen et al., 2010; Cuperus et al., 2010). The RNA polymerase RDR6, together with SUPRESSOR OF GENE SILENCING 3 (SGS3) protein, processes the cleavage products (Vazquez et al., 2004; Montgomery et al., 2008b), yielding long dsRNA molecules that are subjected to DCL4-mediated phased cleavage of 21-nt dsRNA duplexes (Gasciolli et al., 2005; Yoshikawa et al., 2005). One of the strains from each of these duplexes is then loaded into an AGO protein, according to the criteria previously mentioned, in order to promote the silencing of target transcripts (Mi et al., 2008). Four *TAS* genes have been identified in

arabidopsis: *TAS*1, *TAS*2 (both targeted by miR173) (Montgomery et al., 2008b), *TAS*3 (cleaved by miR390) (Allen et al., 2005) and *TAS*4 (targeted by miR828) (Rajagopalan et al., 2006). Interestingly, *TAS*3 seems to undergo a specific pathway that is conserved in several plant species and different from what is observed for other *TAS* genes. This pathway depends on miR390, a 21-nt long conserved miRNA, and requires AGO7 to be bound to the miRNA in order to cleave *TAS*3 transcripts (Adenot et al., 2006; Montgomery et al., 2008a).

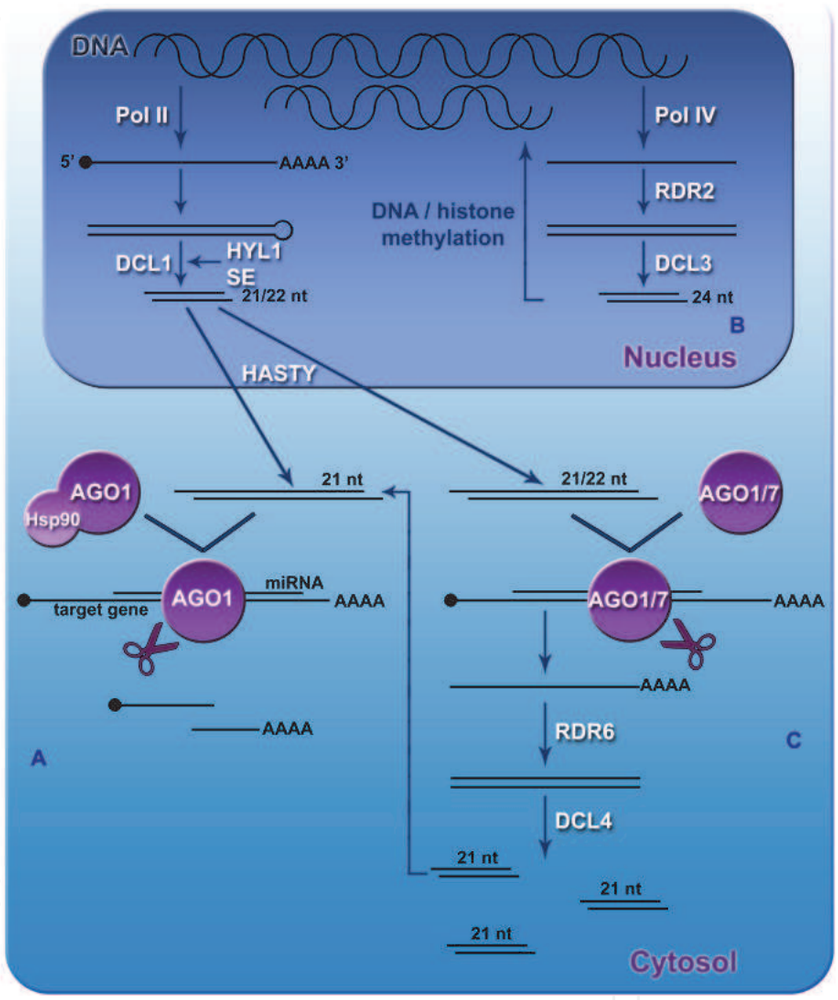


Fig. 1. Biosynthesis and action of the main classes of plant small RNAs. miRNAs are synthesized from stem loop precursors transcribed by Pol II, like protein-coding genes. Most 21-nt miRNAs are loaded into AGO1 and act upon target mRNAs in a sequence dependent manner, by either promoting their cleavage (**A**) or repressing translation (not represented). In the nucleus, siRNAs are synthesized from long dsRNA precursors, transcribed by Pol IV. These small RNAs are mainly loaded into AGO3 and are known to be involved in DNA and histone methylation (**B**). 22-nt long miRNAs are also loaded into AGO1. However, together with miR390, which is 21-nt long and is loaded into AGO7, these miRNAs trigger the biosynthesis of ta-siRNAs by DCL4. The newly formed siRNAs will be loaded into AGO1 and, like miRNAs, promote target cleavage (**C**).

3. Small RNAs and abiotic stress

A large portion of already identified miRNAs are present in several plant species (Reinhart et al., 2002; Bonnet et al., 2004; Wang et al., 2004). Most of these conserved miRNAs are known to have key roles in plant development, targeting a variety of transcription factors (TFs) related to pattern formation and organ differentiation (Llave et al., 2002b; Rhoades et al., 2002; Carrington & Ambros, 2003; Todesco et al., 2010). Lately, however, with high-throughput sequencing techniques becoming more and more accessible, several miRNAs (and other sRNAs) have been identified that seem to be family or species-specific and can be involved in many other processes in plants (Bonnet et al., 2004; Moxon et al., 2008; Szittya et al., 2008; Schreiber et al., 2011; Song et al., 2011). In fact, as the knowledge on small RNAs has broadened and new targets have been identified, it became apparent that, apart from their role in plant development, sRNAs are also involved in other processes in plants (Jones-Rhoades & Bartel, 2004; Sunkar & Zhu, 2004).

Since the early 2000's, several reports have associated sRNAs to abiotic stress responses, showing that post-transcriptional regulation of gene expression plays an important role in these phenomena (reviewed by Covarrubias & Reyes, 2010). The fact that plant miRNAs target transcripts in a sequence-specific manner, allowed Jones-Rhoades and Bartel (2004) to computationally predict targets for a conserved group of these riboregulators. In this work, the authors found that the expression of miR395 depends on sulfate availability, and that some other miRNAs can target transcripts that are potentially involved in responses to abiotic stress (Jones-Rhoades & Bartel, 2004). By then, Sunkar and Zhu (2004), analysed two week-old arabidopsis seedlings, and identified a variety of conserved miRNAs that were differentially expressed upon cold, dehydration, NaCl and ABA treatments. The authors found that, for instance, miR393, miR397b and miR402 were up-regulated in all the implemented conditions. On the other hand, miR389a, which was later found to be related to ta-siRNAs (Allen & Howell, 2010), was down-regulated under all stresses (Sunkar & Zhu, 2004).

Recently, Amor and co-workers (2009) analysed a group of arabidopsis long non-protein coding RNAs (npcRNAs), and found that 22, out of 76, showed differential expression upon water deficit, phosphate starvation and NaCl treatment. Interestingly, these npcRNAs are thought to regulate gene expression in either this long form, or by being precursors for siRNAs and miRNAs (Amor et al., 2009). Moreover, changes in DNA and histone methylation, processes that can be regulated by siRNAs, are known to occur in plants subjected to adverse conditions (Chinnusamy & Zhu, 2009). In fact, it was recently found that in arabidopsis stress-induced transgenerational global genome methylation, and consequent induced stress tolerance, is impaired in *dcl2 dcl3* mutants (Boyko et al., 2010). Together, these observations show that, besides miRNAs, other sRNAs can also be involved in responses to environmental stimuli in plants.

In the following sections we will summarize some of the most important results obtained in this field.

3.1 Osmotic stress: Water deprivation, salinity and abscisic acid

Water deprivation and salt stress alter the osmotic balance inside the cells, and considerable crosstalk is known to exist between responses to both conditions (Table 1) (Shinozaki & Yamaguchi-Shinozaki, 2007). Stomata closure, reduction in cell growth and photosynthesis and higher respiration rates are all general phenomena that can be observed in plants

subjected to osmotic stress (Shinozaki & Yamaguchi-Shinozaki, 2007). Moreover, changes are also registered at the cellular and molecular levels, and several proteins and osmolytes are known to accumulate inside the cells to overcome osmotic changes (Shinozaki & Yamaguchi-Shinozaki, 2007).

In 2007, Zhao and co-workers analyzed the expression of a set of rice miRNAs from seedlings subjected to dehydration stress, induced by PEG treatment, and found that miR169g, miR393 and miR397b were up-regulated in these conditions. Recently, both miR169 and miR393 were also found to be induced by different concentrations of PEG in tobacco (Frazier et al., 2011). However, miR169 was shown to be down-regulated in adult rice plants after water withholding for several days (Zhou et al., 2010), suggesting that sRNA regulation depends on several factors, like the developmental stage and how a certain stress is imposed (Jia et al., 2009b). On the other hand, Zhao and co-workers (2007) showed that, among the miR169 family members, only miR169g seemed to be induced by PEG. In fact, it has been reported that different members of the same miRNA family are often regulated in different ways upon a certain stimuli (Lu et al., 2008). Therefore, it may also be possible, that in both works, different miRNAs were analysed that have distinct expression profiles under similar conditions.

Members of the miR169 family, have also been shown to be induced under high salinity conditions in both arabidopsis and rice (Zhao et al., 2009), and miR393 was also induced after treatment with ABA and NaCl in common bean (*Phaseolus vulgaris*; Arenas-Huertero et al., 2009). These observations suggest that these miRNAs can be involved in general responses to osmotic stress.

The elements of the miR169 family are known to target CCAAT-box Binding Factors (Rhoades et al., 2002), some of which have been shown to be involved in water deficit tolerance in maize (Nelson et al., 2007). However, these TFs were identified in maize as positive regulators of these responses, and their over-expression was shown to improve tolerance to water deprivation and increased yield (Nelson et al., 2007). Therefore, a reduction in the expression of miR169 should be expected in these conditions, and in most cases the opposite is observed. Further studies are necessary to unveil the relation between miR169 and CCAAT-box Binding Factors and their role in responses to osmotic stress.

MiR393, on the other hand, is known to target *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*) transcripts, which are involved in auxin signalling pathways (Jones-Rhoades & Bartel, 2004). MiR393-mediated down-regulation of *TIR1* under stress conditions could result in reduced hypocotyl elongation and lateral root growth, as observed in *tir1* mutants (Ruegger et al., 1998). Strikingly, miR393 was also shown to be induced upon *Pseudomonas syringae* infection and repress bacterial growth, thus establishing a relation between responses to both biotic and abiotic stress conditions (Navarro et al., 2006).

In barrel medic (*Medicago truncatula)*, we have shown that miR408 is highly up-regulated in both shoots and roots of adult plants subjected to water deprivation for one week (Fig. 2) (Trindade et al., 2010). This up-regulation was also recently observed in barley (*Hordeum vulgare*) leaves after dehydration treatment for 4h and 8h (Kantar et al., 2010). MiR408 is known to target plantacyanin-like transcripts in several species, hence it has been related to the control of copper homeostasis in plants (Abdel-Ghany & Pilon, 2008; Trindade et al., 2010). Therefore, it seems that there may be a relation between the reduction of water uptake and copper deficiency, although further studies are needed to confirm this hypothesis.

Despite the intensive crosstalk observed in responses to water deficit and salt stress, not all miRNAs seem to be involved in pathways that are common to both. For instance, different

concentrations of NaCl down-regulated the expression of miR159 and miR167, and induced the expression of miR172 and miR396 in tobacco (*Nicotiana tabacum*) plants (Frazier et al., 2011). On the other hand, these miRNAs were induced after treatment with different concentrations of PEG, in plants grown in the exact same conditions (Frazier et al., 2011). Interestingly, when compared to control plants, most miRNAs exhibited higher differences upon treatment with lower PEG concentrations, than with higher concentrations (Frazier et al., 2011). This observation suggests that miRNAs could be involved mainly in early responses to abiotic stress conditions, like reported for some TFs (Zhu, 2002).

Several water deficit and high salinity-responsive genes are also differentially expressed upon treatments with exogenous abscisic acid (ABA; Table 1) (Zhu, 2002; Nakashima et al., 2009). In fact, it is widely known that responses to osmotic stress comprise both ABA-dependent and ABA-independent pathways (Bohnert & Sheveleva, 1998; Nakashima et al., 2009). For instance, the expression of miR159 was shown to be induced by ABA and drought in arabidopsis, regulating abiotic stress responses in germinating seedlings (Reyes & Chua, 2007).

Moreover, dehydration-responsive elements (DREs) and abscisic acid responsive elements (ABREs), known to be present in the promoter region of water deficit and high salinity-inducible genes (Nakashima et al., 2009), have been identified in the region upstream of several rice *MIR169* genes (Zhao et al., 2007; Zhao et al., 2009). This observation suggests that, besides regulating a large number of TFs, miRNAs can themselves be regulated at the

miRNA	**Regulation**	**Target transcripts**	**References**
miR159	↑ water deficit ↑ ABA ↓ NaCl	*MYB101, MYB33*	Reyes & Chua (2007); Frazier et al. (2011)
miR165/166	↑ cold	*PHABULOSA*, homeobox genes	Zhou et al. (2008); Lv et al. (2010)
miR167	↑ water deficit ↓ NaCl		Frazier et al. (2011)
miR168	↑ water deficit ↑ NaCl	*ARGONAUTE 1*	Liu et al. (2008)
miR169	↓ water withholding ↑ PEG treatment ↑ NaCl	CCAAT-box Binding Factors	Zhao et al. (2007); Zhao et al. (2009); Zhou et al. (2010); Frazier et al. (2011)

miRNA	Regulation	Target transcripts	References
miR171	↑ water deficit ↑ NaCl	*SCARECROW*-like	Liu et al. (2008)
miR172	↑ water deficit ↑ NaCl ↑ cold	*APETALA2*	Frazier et al. (2011)
miR393	↑ water deficit ↑ ABA ↑ NaCl ↑ cold	*TRANSPORT INHIBITOR RESPONSE1*	Sunkar & Zhu (2004); Zhao et al. (2007); Arenas-Huertero et al. (2009); Frazier et al. (2011);
miR396	↑ water deficit ↑ NaCl ↑ cold	*GROWTH REGULATING FACTOR*	Liu et al. (2008); Frazier et al. (2011)
miR397b	↑ water deficit ↑ ABA ↑ NaCl ↑ cold	Laccases	Sunkar & Zhu (2004); Zhao et al. (2007);
miR398	↑ water deficit ↑ ABA ↑ NaCl	*COPPER SUPEROXIDE DISMUTASE, CYTHOCHROME c OXIDASE SUBUNIT V*	Jia et al. (2009a); Trindade et al. (2010)
miR402	↑ water deficit ↑ ABA ↑ NaCl ↑ cold	*ARGONAUTE 2*	Sunkar & Zhu (2004);
miR408	↑ water deficit	*PLANTACYANIN*	Trindade et al. (2010); Kantar et al. (2010)
miR2118	↑ water deficit ↑ ABA	unknown	Arenas-Huertero et al. (2009)

↑ indicates up-regulation; ↓ indicates down-regulation.

Table 1. Conserved miRNAs involved in responses to osmotic and cold stress.

transcriptional level. Interestingly, the over-expression of miR169c in tomato was reported to improve drought tolerance through a reduction in stomata aperture, a process that is controlled by ABA, and consequent reduction of transpiration and leaf water losses (Chinnusamy et al., 2008; Zhang et al., 2011b).

Non-conserved miRNAs have also been reported to be involved in responses to osmotic stress (Table 2). In common bean, the legume-specific miR1514a and miR2119, and the conserved miR2118 were up-regulated upon water deficit and ABA treatments (Arenas-Huertero et al., 2009). Interestingly, miR1514a was predicted to target transcripts for Mn-superoxide dismutase (SOD) and a cysteine protease, while miR2119 was predicted to cleave Aldehyde Dehydrogenase 1 and zinc finger protein mRNAs, among others, (Arenas-Huertero et al., 2009), supporting the hypothesis of their involvement in responses to different types of stresses. Recently, the grass-specific miR1432 and miR1867 (predicted to target phenylalanine tRNA synthetase and a protein from the DUF1242 superfamily, respectively) were also shown to be induced by dehydration in roots and shoots of wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*), respectively (Kantar et al., 2011).

Besides miRNAs, other sRNAs have been shown to have a role in osmotic stress responses. For instance, Borsani and co-workers (2005) identified in arabidopsis a new group of plant siRNAs, known as natural antisense siRNAs (nat-siRNAs), that is formed from overlapping genes (Borsani et al., 2005). Their biogenesis, requires DCL2, RDR6, SGS3 and Pol IV to generate a 24-nt nat-siRNA from D1-pyroline-5-carboxylate dehydrogenase (P5CDH) cleavage, that will set the phase for subsequent cleavage by DCL1 of 21-nt nat-siRNAs from the same transcript (Borsani et al., 2005). In the same work, the authors found that the expression of SRO5 (a transcript of unknown function) is required for the production of nat-siRNAs and that it is induced upon salt treatment. This mechanism represents a unique regulation of salt stress responses, because cleavage of P5CDH leads to accumulation of proline (improving salt tolerance) and reactive oxygen species (ROS), an effect that is counteracted by SRO5 (Borsani et al., 2005). Moreover, the authors mention that they have identified a variety of other siRNAs synthesized from overlapping genes that were only identified under certain abiotic stress conditions, implying that these molecules could have a more generalized role in the responses to environmental constrains.

3.2 Cold stress

Cold stress generally leads to a reduction in metabolic rates and water availability, due to chilling-induced inhibition of absorption (Chinnusamy et al., 2007). Therefore, it is not surprising that crosstalk between water deficit, salt stress and cold has been reported (Table 1) (Bohnert & Sheveleva, 1998).

As for other stress conditions, post-transcriptional regulation of gene expression also plays an important role in response to low temperatures (Chinnusamy et al., 2007), and alternative-splicing in particular is known to have an important role under cold stress conditions (Iida et al., 2004).

In 2008, miR168, miR171 and miR396 were shown to be induced by drought, cold and salt stress in arabidopsis seedlings (Liu et al., 2008), suggesting that miRNAs can be involved in the pathways common to all these stimuli. Following this, Zhou and co-workers (2008) identified four arabidopsis MIRNA genes that are inducible by cold stress, using a computational approach based on transcriptome and promoter analysis data, coupled with experimental validation. Northern blot analysis revealed that miR165/miR166, miR169 and

miR172 were also up-regulated upon cold treatment (Zhou et al., 2008). Some elements of the miR166 family were also found to be up-regulated in similar conditions in rice, while miR168, miR169 and miR171 showed opposite expression profiles (Lv et al., 2010). These observations show once again that miRNA expression upon abiotic stress can be complex and depend on a variety of parameters. Interestingly, most of these conserved cold-regulated miRNAs are known to target TFs with known roles in plant development (Jones-Rhoades & Bartel, 2004), suggesting that miRNA-mediated responses to this kind of stress could be mainly at the structural level.

miRNA	Regulation	Predicted target transcript	Other species	References
pvu-miR1514a	↑ water deficit ↑ ABA	*MnSOD*, *CYSTEINE PROTEASE*	soybean	Arenas-Huertero et al. (2009)
pvu-miR2119	↑ water deficit ↑ ABA	*ALDEHYDE DEHYDROGENASE1*, Zinc finger domain	soybean; barrel medic	Arenas-Huertero et al. (2009)
ttu-miR1432	↑ water deficit ↑ ABA	*PHENYLALANINE tRNA SYNTHETASE*	rice, maize, sorghum	Kantar et al. (2011)
ttu-miR1867	↑ water deficit ↑ ABA	*DUF1242*	rice	Kantar et al. (2011)
osa-miR1320	↓ cold stress	Clathrin assembly, *FUCOSYLTRANSFERASE7*, *REMORIN*	------	Lv et al. (2010)
osa-miR1435	↑ late cold stress	B3 DNA Binding domain, *UDP GLUCOSYLTRANSFERASE*	sorghum	Lv et al. (2010)
osa-miR1876	↓ cold stress	*HISTONE DEACETYLASE6*	------	Lv et al. (2010)
osa-miR1884	↓ cold stress	*ATPase*, *URICASE*, ABA stress-ripening, TMV response-related	------	Lv et al. (2010)
osa-miR1850	↑ oxidative stress	unknown	------	Li et al. (2010)
ptc-miR475a,b	↓ early cold stress	Pentatricopeptide repeat	------	Lu et al. (2008)
ptc-miR476a	↓ cold stress	Pentatricopeptide repeat	------	Lu et al. (2008)

pvu- common bean; *ttu*- wild emmer wheat; *osa*- rice; *ptc*- black cottonwood. ↑ indicates up-regulation; ↓ indicates down-regulation.

Table 2. Non-conserved miRNAs involved in responses to abiotic stress in plants.

A deeper look into the promoter region of some cold-inducible MIRNA genes, revealed an abundance of motifs that are known to be involved in responses to cold, namely W-box (TTGAC), ABRE-core (ACGTGG/TC) and LTRE-core (A/GCCGAC) (Zhou et al., 2008). This observation supports once again the idea that stress-responsive miRNAs can be regulated at the transcriptional level.
Like previously reported for water deficit and salt stress, non-conserved miRNAs are also involved in cold stress responses in plants (Table 2). For instance, a set of rice miRNAs, namely miR1435, miR1876, miR1320, and miR1884, not previously detected in arabidopsis, were recently found to be differentially expressed upon cold treatment (Lv et al., 2010). Moreover, in black cottonwood (*Populus trichocarpa*) the species-specific miR475a,b and miR476a, both predicted to target transcripts coding for pentatricopeptide repeat-containing proteins (PPRs), were shown to be down-regulated in similar conditions (Lu et al., 2008). Interestingly, PPRs were identified in arabidopsis as RNA-binding proteins that seem to be involved in post-transcriptional regulation in mitochondria and chloroplasts (Lurin et al., 2004), suggesting sRNAs can also regulate organelle metabolism.

3.3 Flooding and hypoxia

Flooding can also severely compromise plant growth and productivity, since it is associated to a reduction of oxygen availability to the cells (Licausi, 2010).
The expression of some maize miRNAs was found to be induced after submergence (Zhang et al., 2008b). Strikingly, it seems that different miRNAs are regulated at different time points and in most cases this induction is transient. For instance, miR166, miR167 and miR171 were induced in the early stages, while the expression of miR159 was reduced by this time and further induced after 24h of submergence (Zhang et al., 2008b). Most of these miRNAs were shown to have *cis*-acting elements in their promoter regions that are associated with anaerobic stress, namely AREs (anaerobic responsive elements) and GC-motifs (Zhang et al., 2008b). A member of the miR167 family was also up-regulated in submerged arabidopsis plants, together with elements from the miR156, miR157, miR158, miR161, miR169, miR172, miR391, miR395 and miR775 families (Moldovan et al., 2010). With the exception of miR172, all the other miRNAs exhibited similar expression patterns upon treatment for mitochondrial inhibition, suggesting they are actually hypoxia-inducible miRNAs (Moldovan et al., 2010).
Like previously observed for osmotic stress, other sRNAs can be involved in responses to oxygen deprivation. Recently, Moldovan and co-workers (2010) have shown that several tas-siRNAs derived from the *TAS1*, *TAS2* and *TAS3* loci are also up-regulated in arabidopsis under hypoxia conditions.

3.4 UV radiation and light

According to some recent reports, miRNAs can also be involved in responses to light and radiation stimuli. Zhou and co-workers (2007) gathered data from promoter element analysis and the expression of protein-coding genes predicted to be targeted by miRNAs, and identified *in silico* a group of potentially UV-B inducible arabidopsis miRNAs. Later, using a home-made array, Jia and co-workers (2009) identified miRNAs in black cottonwood that were differentially expressed upon 2h of treatment with the same radiation. As previously predicted for arabidopsis (Zhou et al., 2007), miR156, miR160,

miR165/166, miR167 and miR398 were induced in these conditions, whereas the same pattern was not observed for miR159 and miR393 in both species (Jia et al., 2009a). Moreover, miR169, miR395 and miR472 were down-regulated by UV-B radiation, while the opposite was observed for miR168, miR398 and miR408 (Jia et al., 2009a). These results show that, although several miRNAs are conserved among plant species, their regulation upon certain stimuli can actually be different.

Also, in both arabidopsis and common aspen (*Populus tremula*), the majority of the selected miRNAs exhibited stress and light-inducible *cis*-acting elements, like GT-1 site (GGTTAA) and I-boxes (GATAAGA), upstream of their coding genes (Zhou et al., 2007; Jia et al., 2009a). Besides being regulated by UV radiation, some miRNAs also seem to be regulated by the photoperiod. For instance, miR167, miR168, miR171 and miR393 were shown to accumulate during the light period in arabidopsis (Sire et al., 2009). This expression pattern was absent in plants grown under continuous light, showing that these miRNAs were actually induced by light and not by the circadian clock (Sire et al., 2009).

3.5 Oxidative stress

In plants, oxidative stress usually emerges as a secondary effect of other stress conditions and is highly responsible for losses in crop productivity (Bartels & Sunkar, 2005; Sunkar et al., 2006). To overcome the higher production of ROS upon stress stimuli, plants have developed enzymatic and non-enzymatic detoxification strategies (Bohnert & Sheveleva, 1998), and during the last decade, several works have reported the involvement of sRNAs in these mechanisms.

In an attempt to mimic oxidative stress, Li and co-workers (2010) treated rice seedlings with different concentrations of H_2O_2, and found that the conserved miR169, miR397 and miR827 were up-regulated in these conditions, while miR319a and miR528 were down-regulated. Moreover, the authors identified a rice-specific miRNA, miR1850, that was induced by the applied stress (Li et al., 2010), providing extra evidence to the involvement of miRNAs in both general and species-specific mechanisms of abiotic stress response.

The expression of miR398 has been shown to be repressed upon oxidative stress (Sunkar et al., 2006; Jagadeeswaran et al., 2009), and, as expected, it is down-regulated under several adverse conditions, like salt stress, ozone fumigation and even infection with *Pseudomonas syringae* (Jagadeeswaran et al., 2009). Besides, targeting cytochrome c oxidase (Jones-Rhoades & Bartel, 2004; Trindade et al., 2010), this miRNA is known to target both cytosolic and plastidic Cu/Zn superoxide dismutases (CSD1 and CSD2, respectively) (Jones-Rhoades & Bartel, 2004; Sunkar et al., 2006), that are responsible for the conversion of O_2^- to H_2O_2 (Kliebenstein et al., 1998). Therefore the reduction in the levels of miR398, leads to an increase in CSD transcripts, thus protecting the cells against oxidative damage (Sunkar et al., 2006).

However, the expression of miR398 is not always decreased under abiotic stress conditions. In adult barrel medic plants subjected to water deficit for one week, this miRNA was highly up-regulated in both shoots and roots (Fig. 2) (Trindade et al., 2010), a result that was also recently observed in both PEG-treated tobacco (Frazier et al., 2011) and dehydrated wild emmer wheat (Kantar et al., 2011). A similar expression profile had previously been observed after sucrose addition in arabidopsis (Dugas & Bartel, 2008) and in common aspen subjected to short time treatments with ABA, NaCl and UV-B radiation (Jia et al., 2009a). Interestingly, it was recently shown that the expression of this miRNA not only depends on the type of stress imposed, but it also seems to oscillate with the degree of stress (Frazier et al., 2011).

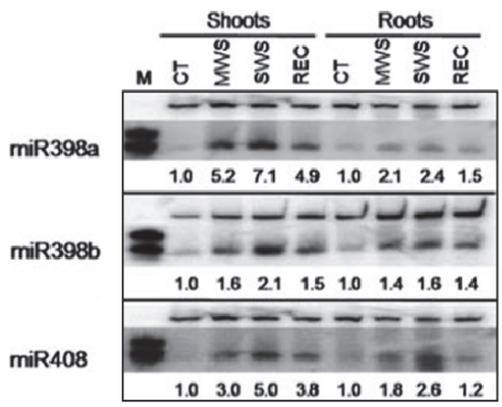


Fig. 2. Northern blot analysis of the expression of miR398a, miR398b and miR408 in both shoots and roots of 8 week-old *Medicago truncatula* plants subjected to different degrees of water deficit: CT- control; MWS - moderate water deficit; SWS- severe water deficit; REC- recovery; M-microRNA size marker from New England Biolabs (the darker bands correspond to 24 and 21 nt). Small nuclear RNA U6 was used as a loading control (Trindade et al., 2010).

Besides having a role in response to oxidative stress, miR398, along with other miRNAs, has been associated to the maintenance of copper homeostasis in plants (Abdel-Ghany & Pilon, 2008). Overall these results seem to suggest that this miRNA can be regulated in opposite ways by at least two distinct pathways in order to protect the cells from damage. One pathway seems to be dependent on the copper status of the plant, while the other seems to be related to oxidative stress. Some authors have hypothesized that the induction of miR398, and consequent degradation of transcripts of copper proteins, could be a way of saving copper for vital proteins like plastocyanin, known to be involved in the electron transport chain of the photosynthetic pathway (Abdel-Ghany & Pilon, 2008). According to Dugas and Bartel (2008), the induction of this miRNA, and further reduction of CSD transcripts, under these conditions could be caused by a reduction in ROS production as a consequence of some photosynthetic impairment. It could also be that different MIR398 genes are being regulated in different ways, like previously proposed for MIR159 (Reyes & Chua, 2007). A deeper analysis on the expression pattern and the promoter regions of each miR398 family member could shed some light on this matter.

3.6 Hormone signalling

Plant hormones are also known to play a role in abiotic stress responses. As mentioned above, many genes that have been shown to be induced by drought, salt stress and cold are also regulated upon exogenous ABA treatments (Zhu, 2002).

Besides ABA, other phytohormones like auxins, cytokinins, gibberellins, among others, can also be directly or indirectly involved in responses to environmental stimuli. Over the last years, several reports have shown that some sRNAs can be involved in these signalling pathways, either by being regulated by these hormones, or by targeting hormone-responsive transcripts (Guo et al., 2005; Reyes & Chua, 2007). In 2005, Sorin and co-workers reported that *ago1* mutants were practically unable to form adventitious roots and that this abnormal development was related to impairment in the auxin metabolism, suggesting an

involvement of miRNAs in these processes (Sorin et al., 2005). Previously, it had also been shown that *hyl1* mutants exhibited altered sensitivity to different hormone treatments (Lu & Fedoroff, 2000). Additionally, some abiotic stress-inducible miRNAs exhibit hormone-responsive *cis*-acting elements, like ABRE, gibberellic acid (GA3)-responsive element (GARE), methyl jasmonate-responsive element (CGTCA motif) and salicylic acid-responsive element in their promoter regions (Liu et al., 2009; Lv et al., 2010). Taken together, these data strongly suggest that there is an involvement of plant hormones in sRNA-mediated responses to abiotic stress in plants.

Auxins, for instance, were recently shown to participate in responses to salt and oxidative stresses in arabidopsis through regulation of the redox metabolism, in which TIR1 and AFB2 auxin receptors, known to be targeted by miR393 (Jones-Rhoades & Bartel, 2004), seem to be involved (Iglesias et al., 2010). Also, some light and cold-inducible miRNAs are known to be involved in the regulation of auxin signalling pathways (Zhou et al., 2007; Zhou et al., 2008).

In rice, miR167 and miR169 were down-regulated by ABA and miR168 and miR169 were down and up-regulated, respectively, after auxin treatment (Liu et al., 2009). miR167 is known to target ARF6 and ARF8, which are regulators of female and male reproduction (Wu et al., 2006). These observations led to the hypothesis that ABA-induced down-regulation of this miRNA could be related to early reproduction, a phenomenon that is generally observed in plants subjected to stress conditions (Liu & Chen, 2009). Moreover, there seems to be a relation between auxin and ABA signalling pathways, in which miR167 is apparently involved. Also, miR319 was shown to be up-regulated by both GA_3 and ABA, and down-regulated by cytokinins (Liu et al., 2009), suggesting that miRNAs can be involved in processes of crosstalk between different phytohormones and between those and abiotic stress conditions.

Upon certain stress stimuli, plants can respond by altering their growth and development, in order to avoid or reduce stress-inducible damage. Auxins are known to be involved in the regulation of plant development, and could play an important role in these mechanisms (reviewed by Vanneste & Friml, 2009). For instance, the previously mentioned up-regulation of miR393 under stress conditions, and consequent regulation of auxin signalling pathways, has been related to a reduction of plant growth observed in these situations (Sunkar & Zhu, 2004).

Abnormal root growth and development has also been observed in several plant species subjected to osmotic stress (Bartels & Sunkar, 2005). Recently, it was found that lateral root growth in arabidopsis depends on the tight feedback regulation between miR390, involved in the production of *TAS3*-derived ta-siRNAs, and an auxin responsive factor (ARF4), known to be targeted by these siRNAs (Allen et al., 2005; Marin et al., 2010). These results show that siRNAs can be involved in hormone signalling pathways during plant development as well, suggesting they could also regulate hormone-mediated responses to abiotic stress.

As mentioned earlier, miR393 was found to be involved in responses to bacterial infection in arabidopsis by regulating a group of auxin receptors (Navarro et al., 2006). Recently, other miRNAs, known to target hormone-related transcripts, were shown to be differentially expressed upon infection with different bacterial strains (Zhang et al., 2011a). These observations show that the relation between sRNAs and phytohormones can go beyond plant development and abiotic stress tolerance.

4. Regulation of small RNA pathways by abiotic stress

RNA-mediated regulation of gene expression as response to abiotic stress conditions depends, not only on the expression of sRNAs and their targets, but it also seems to rely on the regulation of the proteins involved in their biosynthesis. In 2003, Szittya and co-workers have shown that virus and transgene-induced RNA silencing was dependent on temperature. The authors found that the accumulation of siRNAs, but not of miRNAs, was lower under cold stress and speculated that the activity of some DCL enzymes could be affected in these conditions (Szittya et al., 2003). Recently, in our laboratory we have shown that several *AGO* and *DCL* genes are differentially expressed in barrel medic adult plants subjected to water deficit (Capitão et al., 2011).

One striking feature of miRNA-mediated gene silencing in plants is that it is subjected to feedback self-regulation. Not only is *DCL*1, which is related to the biogenesis of most plant miRNAs, targeted by miR162 (Xie et al., 2003), but *AGO*1 is also known to stabilize miR168 and, simultaneously, be targeted by this miRNA (Rhoades et al., 2002; Vaucheret et al., 2004; Vaucheret et al., 2006). Strikingly, both miR162 and miR168 have been reported to be differentially expressed in abiotic stress conditions, like hypoxia (Zhang et al., 2008b), UV-B radiation (Jia et al., 2009a), water deficit (Zhou et al., 2010) and salt stress (Ding et al., 2009). This observation supports the idea that the expression of sRNAs, and therefore of target genes, can be regulated indirectly through the regulation of key enzymes of the biosynthesis pathway.

Interestingly, Moldovan and co-workers (2010), have reported a reduction in 24-nt long sRNAs and an increase in 21-nt long species in arabidopsis plants subjected to hypoxia, when compared to controlled conditions. This observation suggests that other enzymes, besides DCL1 and AGO1 can be regulated by environmental conditions. In fact, *hen1*, *hyl1, hsty* and *se* mutants are characterized by hypersensitivity to ABA, leading to a lower tolerance to osmotic stress (Lu & Fedoroff, 2000; Zhang et al., 2008a). Also, the expression of RDR6, known to be involved in the biosynthesis of ta-siRNAs and nat-siRNAs, was recently shown to be induced by ABA (Yang et al., 2008).

Taken together, these results support the notion that altering sRNA biosynthesis pathways can affect the response to environmental conditions in plants. Whether the regulation of these proteins can also be mediated by other sRNAs, like it was found for DCL1 and AGO1, is yet to be investigated.

5. Concluding remarks

Despite the large amount of work published over the last years on sRNA-mediated regulation of gene expression under abiotic stress conditions, a lot of information is still missing on the functionality of most sRNAs. So far, this type of studies has been undertaken for just a few conserved miRNAs, several of them related to nutrient status in plants (reviewed by Sunkar et al., 2007).

In fact, the diversity of sRNAs, the observed redundancy in some biosynthesis pathways and their feedback regulation, unveil a higher complexity than previously thought when RNA silencing mechanisms were first identified in plants. Also, as reviewed in this manuscript, the expression of sRNAs seems to depend on a wide variety of factors, from plant species, to developmental stage, to growth conditions, among others, making it hard to fully understand their role under abiotic stress conditions.

Additionally, some recently identified non-conserved miRNAs seem to have no specific targets, or their experimental validation has proven difficult. In fact, computational tools for

target prediction have been reported to yield a high percentage of false positives for these miRNAs (Moxon et al., 2008), which, at some extent, can account for that limitation. Nevertheless, a few of them were already shown to be differentially expressed in abiotic stress conditions, and are probably involved in more specific and less studied response mechanisms.
In 2008, Addo-Quaye and co-workers have reported the sequencing of arabidopsis degradome, which is a technique that allows the high-throughput sequencing of mRNAs without the 5'-end cap, including degraded transcripts and targets of sRNA-mediated cleavage. Using this technique that does not rely on *in silico* predictions, the authors were able to confirm several previously identified targets, identify new targets for some miRNAs and ta-siRNAs, and also to identify new *TAS* transcripts (Addo-Quaye et al., 2008). The sequencing of the degradome is now being adopted for other plant species (Addo-Quaye et al., 2009; Pantaleo et al., 2010) and is expected to retrieve interesting new information on the function and regulation of sRNAs in plants subjected to different conditions.
Phytohormones and stress-inducible TFs seem to play an important role in regulating the expression of miRNAs under abiotic stress conditions, in a similar way to what happens with protein-coding genes. Supporting this idea is the common observation that most miRNAs, besides targeting several TFs, also have stress-related *cis*-acting elements in their promoter regions. These data unveil a complex interaction between transcriptional and post-transcriptional regulators of gene expression that seems to be crucial to fine-tune responses to different environmental stimuli.
Additionally, when it comes to abiotic stress tolerance, most studies on sRNA-mediated regulation of gene expression have been performed inside laboratories, in highly controlled systems. However, in the wild, plants are hardly found in these conditions, and are usually subjected to different types of stresses at the same time. Interestingly, according to several studies, the responses to a situation of multiple simultaneous stresses can actually be different from the responses observed when two or three types of stress are induced separately (reviewed by Mittler, 2006). Therefore, some of the results already verified might even not be observed in the field, and care must be taken when analysing the function of sRNAs in these processes.
Over the last years, it has become clear that sRNAs play an important role in abiotic stress responses in plants. Moreover, they have also been related to pathogen infection and symbiosis (Navarro et al., 2006; Branscheid et al., 2010), revealing their importance in biotic stress conditions as well. Nevertheless, although a lot has been said on the biosynthesis and function of sRNAs, at the moment it seems to be just the tip of the iceberg and exciting new developments are expected in the near future.

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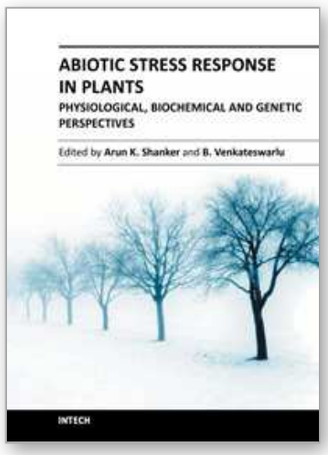
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Plants, unlike animals, are sessile. This demands that adverse changes in their environment are quickly recognized, distinguished and responded to with suitable reactions. Drought, heat, cold and salinity are among the major abiotic stresses that adversely affect plant growth and productivity. In general, abiotic stress often causes a series of morphological, physiological, biochemical and molecular changes that unfavorably affect plant growth, development and productivity. Drought, salinity, extreme temperatures (cold and heat) and oxidative stress are often interrelated; these conditions singularly or in combination induce cellular damage. To cope with abiotic stresses, of paramount significance is to understand plant responses to abiotic stresses that disturb the homeostatic equilibrium at cellular and molecular level in order to identify a common mechanism for multiple stress tolerance. This multi authored edited compilation attempts to put forth an all-inclusive biochemical and molecular picture in a systems approach wherein mechanism and adaptation aspects of abiotic stress are dealt with. The chief objective of the book hence is to deliver state of the art information for comprehending the effects of abiotic stress in plants at the cellular level.

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