Chapter 3
Signaling by MicroRNAs in Response to Abiotic Stress

Guadalupe Sosa-Valencia, Alejandra A. Covarrubias, and José Luis Reyes

Introduction

As part of a large repertoire of strategies to cope with environmental variations, plants have chosen to include the use a vast array of non-coding RNAs to regulate gene expression. Among these, microRNAs (miRNAs) have been extensively studied in recent years and found to participate in numerous phenomena in plants ranging from metabolic responses to developmental decisions. Thus, it is not surprising that they have also been recruited to participate in pathways selected to counteract the adverse effects of biotic and abiotic stress. In this chapter we will first introduce the general pathways for maturation of microRNAs followed by their mechanisms of action. This overview will provide the context to present examples of microRNA involvement in stress signaling and will provide us with the framework to suggest potential points of regulation by stress signals. We also present recent advances in the field originating from genome-wide analyses and other data suggesting future directions towards a better understanding of the role of microRNAs in modulating plant responses to abiotic stress.

MicroRNAs and Other Small RNAs

MicroRNAs were first identified in animals as result of the characterization of developmental decisions in *Caenorhabditis elegans* (Lee et al. 1993). Almost a decade later, they were shown to be present in animals in large numbers (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001), and eventually in

G. Sosa-Valencia • A.A. Covarrubias • J.L. Reyes (✉)
Department of Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Av. Universidad 2001, Cuernavaca, Morelos C.P. 62210, Mexico
e-mail: jlreyes@ibt.unam.mx

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plants (Llave et al. 2002a; Rhoades et al. 2002). Subsequent studies in Arabidopsis and several plant systems have revealed numerous other small RNAs including small interfering RNAs (siRNAs) which comprise the majority of the small RNA population and have been implicated in various pathways of gene silencing (Chen 2010). Among these, *trans*-acting siRNAs (tasiRNAs), natural antisense siRNAs (nat-siRNAs) or repeat-associated siRNAs (rasiRNAs) represent alternate small RNAs involved in silencing pathways employing additional members of the silencing machinery for their biogenesis and function. While current data on their activities could shed light into the intricacies of microRNA functions, interested readers are encouraged to turn to recent reviews in these subjects (Chen 2010; Law and Jacobsen 2010; Vazquez et al. 2010). Up until now, these small RNAs have not been directly linked to stress responses or stress signaling except for a handful of cases. Thus, these examples will be mentioned later on to mark the potential for other pathways to influence stress responses. In contrast, numerous studies have underscored the contribution of microRNAs to stress responses and we will focus mainly in these RNA molecules.

MicroRNA Biogenesis and Action

Maturation of MicroRNAs

While biogenesis of animal microRNAs is similar to that present in plants, there are certain differences that will be mentioned as we describe the pathway in plants (Fig. 3.1). Plant microRNA genes (*MIR* genes) are in general found as independent transcription units with their own regulatory promoter sequences. Transcription by RNA Polymerase II is the norm and transcripts are in general capped and polyadenylated (Lee et al. 2004; Parizotto et al. 2004). Soon after transcription, the pre-miRNA adopts a characteristic hairpin secondary structure and is sequentially recognized by the cap binding complex components CBP20 and CBP80 (Kim et al. 2008) and DAWDLE (Yu et al. 2008) to be followed by binding and processing by DCL1 (a member of the DICER-LIKE family of proteins) aided by SERRATE (SE) and HYPONASTIC LEAVES 1 (HYL1) (Kurihara and Watanabe 2004; Vazquez et al. 2004; Yang et al. 2006). The product is a double-stranded duplex of 20–24 nts in length with a 5'-phosphate and a two-nucleotide overhang at the 3'-end. This RNA duplex is methylated at the 2'OH position of the last ribose at the 3'-ends of each strand by the HUA-ENHANCER 1 (HEN1) methyltransferase activity (Park et al. 2002; Yu et al. 2005). The RNA duplex is subsequently transported to the cytoplasm via HASTY, a Ran-GTPase homologous to mammalian Exportin 5 (Park et al. 2005), and recruited to an ARGONAUTE 1-containing complex (RISC) where one of the two strands is selected to represent the mature miRNA while the other strand, known as the microRNA* strand is rapidly degraded (Baumberger and Baulcombe 2005; Vaucheret et al. 2004).

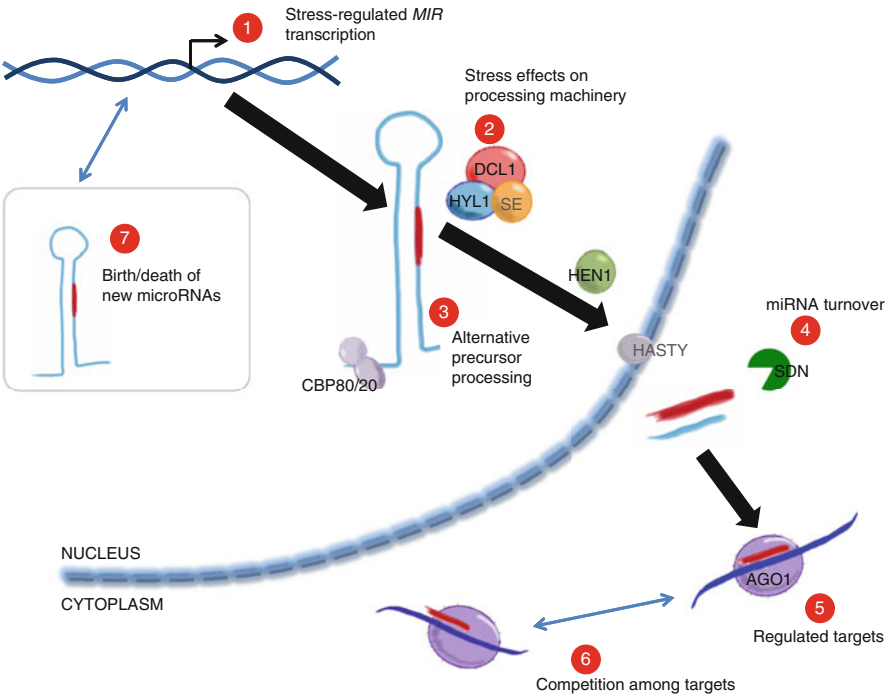

Fig. 3.1 Stress signals affecting microRNA biogenesis and activities. The diagram shows the microRNA biogenesis pathway in plants with selected biogenesis factors that have been found in different studies to be affected by abiotic stress (see main text). Elements influencing the outcome of the pathway discussed in the text are numbered. (*1*): Regulated transcription in response to stress (either repression or induction, as suggested by microarray and high-throughput sequencing experiments); (*2*): mutant analysis has revealed factors in the biogenesis pathway that are required for adequate stress responses (names in *black letters*, factors in *grey* have not been linked to stress); (*3*): alternative processing of microRNAs precursor (sequence variants or additional DCL products) may generate additional functional small RNAs; (*4*): microRNA (and target) turnover could influence the final result of microRNA regulation; (*5*): the MicroRNA-regulated transcript products are proposed to directly contribute to stress responses; (*6*): the presence of multiple targets for the same microRNA could compete for microRNA binding; (*7*): acquisition of novel microRNAs in the genome can reshape how stress responses are modulated over evolutionary time

Although this general biogenesis pathway applies to most conserved plant microRNAs studied so far, there are a few interesting examples deviating from the canonical process, where the differences may be related to precursor features and/or processing, or other associated factor activities that result in the production of additional small RNAs with the properties of microRNAs. In either case, it can be speculated that variations can be due, or at least influenced by, environmental input and by stress signals. For instance, a few reports where high-throughput sequencing has been used to determine the microRNA profile under different growth conditions have found that the otherwise unstable and low abundance

microRNA* sequences are enriched up to detectable levels and sometimes even more abundant than the annotated and functional microRNA strand (Devers et al. 2011; Wong et al. 2011). Interestingly, in response to phosphate deficiency, there was an increased accumulation of particular microRNA* sequences in Arabidopsis that did not completely correlate with an increase in the corresponding microRNAs (Pant et al. 2009). These examples raise the possibility of their participation and functionality under stress conditions, and suggest that microRNAs* could participate by regulating expression of other mRNAs, as has been elegantly demonstrated in *Drosophila* (Okamura et al. 2008). Moreover, recent discoveries also originating from small RNA sequencing strategies in different plant species have shown that microRNA precursors possess the potential to generate more that one RNA duplex processed by a DCL protein. First, variants differing in a few nucleotide positions from the canonical mature microRNA sequence, but originating from the same precursor, could potentially target mRNAs with the complementary sequence [for a recent example see (Jeong et al. 2011)]. Alternatively, DCL proteins could sequentially process long microRNA precursors to generate other small RNAs. Selected examples have been described in *Populus trichocarpa*, *Phaseolus vulgaris* and Arabidopsis that could potentially be alternative DCL products with biological activity (Contreras-Cubas et al. 2012; Lu et al. 2008; Zhang et al. 2010).

An interesting example is the miR159 and related miR319 precursor. The majority of the microRNA precursors are recognized from the base of the precursor, where the transition from single to double-stranded RNA is a signal for processing by the DCL1, SE and HYL1 factors (Mateos et al. 2010; Song et al. 2010; Werner et al. 2010). In pre-miR159/319, DCL1 starts cleavage of the precursor from the terminal loop at 20-21nts intervals, until it reaches the position of the mature miR159/319 sequence. In Arabidopsis and *Physcomitrella patens* the intermediate sequences are of extremely low abundance, only detectable by deep sequencing (Addo-Quaye et al. 2009; Bologna et al. 2009). In contrast, other species show higher abundances of one of the equivalent small RNAs, designated as miR159.2, suggesting its functionality. This is consistent with the fact that *P. vulgaris* miR159.2 can be recruited to AGO1-containing complexes and is functional in a heterologous system. Interestingly, the abundance of *Phaseolus* miR159a and miR159.2 did not always correlate under stress conditions (Contreras-Cubas et al. 2012), and possibly in other plants including soybean, rice and maize as well (Li et al. 2011c), suggesting that its abundance is regulated under particular environmental situations (see section *Additional Elements Affecting MicroRNA Activities*).

RISC Activity

Mature microRNAs are recruited to a cytoplasmic multi-protein complex known as RNA-Induced Silencing complex (RISC) composed of several protein factors, but

most importantly by the catalytic subunit, a member of the ARGONAUTE (AGO) protein family. *Arabidopsis thaliana* contains ten *AGO* genes, while *Oryza sativa* has eighteen. These diversity has been attributed to specific functions carried out by individual family members (Vaucheret 2008). *In vitro* reconstitution experiments, genetic analysis and deep sequencing of small RNAs specifically associated to AGO proteins, all point to AGO1 as the major AGO protein controlling and executing microRNA activity in plants (Baumberger and Baulcombe 2005; Mi et al. 2008; Vaucheret et al. 2004). A notable exception is miR390, which is bound by AGO7 in the Arabidopsis pathway to initiate processing of TAS3 transcripts, leading to phased-processing of a double-stranded RNA intermediate by DCL4 for tasiRNA production (Montgomery et al. 2008). In the few well-characterized examples available, tasiRNAs are subsequently recruited to AGO-containing complexes to negatively regulate gene expression (Chen 2010), however their possible involvement in pathogen responses has only recently been reported in legumes (Zhai et al. 2011).

A microRNA within the RISC complex recognizes its target mRNA through extensive RNA:RNA base-pairing. This interaction can result in one of two outcomes: AGO1 catalyzes cleavage of the mRNA at the position opposite to nucleotides 10th and 11th of the microRNA as long as these bases are involved in a Watson-Crick base-pairing interaction (Llave et al. 2002b). Alternatively, the mRNA is not degraded but instead the microRNA function is redirected to translational inhibition of its target mRNA. Current evidence indicates that AGO- and microRNA-containing complexes directing translation inhibition are recruited to translating polysomes (Brodersen et al. 2008; Lanet et al. 2009). Although this activity has been observed in selected examples, the extent of its participation during stress responses has not been determined yet.

Due to the extensive base-pairing that has been observed between plant microRNAs and their target transcripts, several studies have widely uncovered the regulatory mechanisms of several microRNAs in the model plant *A. thaliana* using a wide variety of approaches, including genome-scale bioinformatical prediction of target mRNAs (Jones-Rhoades and Bartel 2004) and high throughput sequencing approaches designed to identify mRNAs cleaved by microRNA activity, commonly known as 'degradome' or 'PARE' (for Parallel Analysis of RNA Ends) (Addo-Quaye et al. 2008; German et al. 2008).

Thus, it would seem that finding the regulated target for a given plant microRNA has become a routine task, with degradome data available for Arabidopsis (Addo-Quaye et al. 2008; German et al. 2008), as well as a variety of plant species (Devers et al. 2011; Li et al. 2010; Pantaleo et al. 2010; Song et al. 2011), which include specific organs, developmental stages, and in our particular case of interest, abiotic stress conditions, such as *Medicago* plants exposed to mercury (Zhou et al. 2012), or *P. euphratica* leaves subjected to drought (Li et al. 2011a), to name some recent reports.

Although these studies provide useful information about those mRNAs subjected to microRNA regulation, however, in addition to the identification of the relevant transcripts and its association to a particular cellular process, it has become increasingly evident that other factors play important roles in modulating the microRNA:target interactions. Features such as the half-life of the microRNA

target and the microRNA itself; the competition between one or more coding and non-coding transcripts for miRNA binding; the differences between spatial, temporal and condition-specific expression of both target and microRNA is achieved; or the birth of new miRNAs during evolution and their incorporation into novel regulatory pathways. Although each of these factors could, in principle, be influenced by input from adverse environmental conditions and therefore affect the output of the microRNA pathway, we only know of a few cases, which will be highlighted as we briefly overview these factors.

Additional Elements Affecting MicroRNA Activities

Half-Life of the MicroRNA Target and the MicroRNA Itself

Like other RNA molecules, the microRNA and its mRNA target have a half-life, which is defined by the contribution of different processes, such as the rate of transcription, the rate of processing (i.e. pre-microRNA processing or RNA splicing for mRNAs), or their degradation by exonucleases. In plants, the major 3' to 5' exoribonuclease family for small RNAs is encoded by the *SMALL RNA DEGRADING NUCLEASE* (*SDN*) genes (Ramachandran and Chen 2008). The degradation process is thought to be facilitated by post-transcriptional uridylation of small RNA 3'-ends, possibly mediated by a homolog of MUT68, a *Chlamydomonas reinhardtii* nucleotidyltransferase involved in microRNA and siRNA uridylation (Ibrahim et al. 2010). Interestingly, the levels of Arabidopsis *SDN* transcripts were found to be altered by ABA and drought and even more so by extreme temperatures (Laubinger et al. 2010), suggesting that these stress signals affect the half-life of microRNAs through modulation of the activity of SDN enzymes. In contrast, the XRN2 and XRN3 exoribonucleases in Arabidopsis promote degradation of the loop sequence of microRNA precursor without affecting the mature levels of microRNAs (Gy et al. 2007). Whether lack of these nucleases results in altered responses to stress remains to be addressed.

Similarly, the half-life of the target mRNA may also affect its regulation by microRNAs: it has been shown in animal cells that short-lived mRNAs may be more difficult to process by microRNAs, and conversely a stable target may be more easily recognized and processed by microRNAs, however the relative abundance of the mRNA when compared to that of the microRNA should also be considered (Arvey et al. 2010; Larsson et al. 2010). Ultimately, the microRNA and its targeted transcript should be present in the same cell, at the same time. Finally, in addition to tissue-specificity and developmental stage changes affecting microRNA and/or target mRNA gene co-expression, there is convincing evidence that several small RNAs are transported through the phloem and spread systemically (Yoo et al. 2004), thus changing the distribution of microRNAs and their activity on their targets.

For example, miR399 is induced in response to low-phosphate conditions (see discussion below) and transported from the roots to shoots, however the significance of this movement is not fully understood (Pant et al. 2008).

Competition Between One or More Coding and Non-Coding Transcripts for miRNA Binding

Unlike animal microRNAs that are known each to have several transcripts as targets (Lim et al. 2005), plant microRNAs usually regulate only a few mRNAs, a feature possibly reflecting the extensive microRNA:mRNA base-pairing occurring in plants but contrasting with the limited base-pairing observed in animals (Axtell et al. 2011). The presence of a target mRNA could potentially influence the expression of another transcript already under microRNA control by recruiting the microRNA-loaded RISC and releasing the formerly regulated transcript from its inhibited expression. An example of such regulation in plants occurs in the context of phosphate starvation. miR399 is induced upon phosphate limitation and recognizes the PHO2 mRNA, encoding a E2 ubiquitin conjugase, an important negative regulator of phosphate deprivation responses (Bari et al. 2006). miR399-guided cleavage of PHO2 mRNA allows for proper phosphate limitation responses. To modulate miR399 activity, Arabidopsis and other plants induce the expression of members of the *INDUCED BY PHOSPHATE STARVATION* (IPS) 1 family of RNAs. These transcripts lack an open reading frame, however they contain a sequence partially complementary to miR399, which serves to sequester the available miR399 allowing for accumulation of PHO2 transcripts. IPS1 transcripts, also known as miRNA target mimics effectively compete with PHO2 mRNA for binding of miR399 to modulate its activity and achieve phosphate homeostatic conditions (Franco-Zorrilla et al. 2007). One can envisage that changes in the accumulation patterns of RNA molecules containing microRNA binding sites will affect the regulation of other mRNAs by the same microRNAs. The response to phosphate limitation has acquired this regulatory module and it is expected that other signaling pathways have gained it too. Additionally, we can expect to see competition among different coding target mRNAs for a particular microRNA, effectively establishing communication between mRNAs through a microRNA language, as recently proposed (Salmena et al. 2011).

Birth of New MicroRNAs

The formation of novel *MIR* genes and their subsequent insertion as regulators of target transcripts is a phenomenon that continuously shapes the regulatory mechanisms of plants. Although there are different models to explain the birth of new microRNAs, including a duplication event generating a partially inverted-repeat copy, an interesting model involves the recruitment of miniature inverted

repeat elements (MITEs) that become substrates for DCL proteins (Axtell et al. 2011). If the small RNAs generated provide a selective advantage, a novel microRNA could arise. Interestingly, two MITE-derived small RNAs, siR441 and siR446 in rice have been shown to respond to ABA and drought and act as positive regulators of ABA signaling and abiotic stress responses, possibly regulating target mRNAs (Yan et al. 2011). Newly emerged microRNAs may not always possess a regulatory target *ab initio*, thus they may be subjected to selection, and possible extinction if they do not provide an advantage to the plant.

What Is Known About MicroRNAs and Stress?

In principle, given that the microRNA pathway can regulate several processes within the cell, and it can be used to finely regulate gene expression, the effect of stress at multiple levels of this pathway could profoundly influence its outcome. For example, microRNA expression could be specifically regulated by stress to modulate accumulation levels of its downstream mRNA targets, which in turn directly contribute to stress responses. In these cases, defining microRNA accumulation patterns under stress conditions as well as their relevant mRNA targets is essential to understand microRNA contribution to stress responses. Several studies have revealed specific microRNA families involved in stress responses, mainly through the use of high throughput sequencing or microarray analysis (see section *Genome-Wide Analyses* below). Such evidence indicates that microRNAs participate in response to a wide variety of external stimuli including drought, salt, cold, heat and other forms. Although certain microRNA families are repeatedly found (i.e. miR393, miR398, miR169), several studies report novel microRNAs, specifically found in different plant species that could be contributing to stress responses according to their adaptive history.

An additional effect of abiotic stress upon plant processes could be through direct modulation of the factors participating in the microRNA pathways, such as the biogenesis proteins or pathway effectors. Such regulation would influence microRNA activities at the global level and thus would have a distinct and potentially broader consequence on cell processes during stress. Evidence for an effect at this level of microRNA activity has come from the analysis of mutations in factors involved in microRNA metabolism and the corresponding alterations in responses to stress. A brief description of recent advances in these areas will be presented in the following sections.

MicroRNAs Involved in Stress Responses

The specific roles of individual microRNAs during stress responses have been documented, revealing that microRNAs can regulate transcription factors,

enzymatic activities or other regulators. The analysis of each of those microRNAs found in Arabidopsis and conserved in other plant species has provided clues as to how plants respond to different stress stimuli. In Arabidopsis seedlings, miR159 was found to accumulate in response to ABA and drought, it controls the levels of two transcription factors, MYB33 and MYB101 that act as positive regulators of plant responses during germination (Reyes and Chua 2007). In contrast to miR159, the levels of miR169 decreased under stress conditions, which allowed for accumulation of its target mRNA encoding NFYA5, a subunit of the trimeric transcription factor Nuclear factor-Y (NFY), and consequently mediating ABA-dependent responses that included stomata closing and a reduction of leaf water loss (Li et al. 2008). Accumulation of miR395 is induced by low-sulfate conditions in the medium (Jones-Rhoades and Bartel 2004), and mediates sulfate homeostasis by cleaving the transcripts encoding the low-affinity sulfur transporter SULTR2;1 and the ATP sulfurylases APS1 and APS4 (Allen et al. 2005; Jones-Rhoades and Bartel 2004). In this case and for miR398, we observe that microRNA regulation is directed towards enzymatic activities and not transcription factors. miR398 regulates the mRNA for the copper-dependent superoxide dismutase CSD1 and cytochrome C oxidase subunit V (Jones-Rhoades and Bartel 2004). It participates in numerous response pathways, including those for oxidative stress, water deficit, salt stress, ultraviolet stress, copper and phosphate deficiency, among others (Zhu et al. 2011). As mentioned earlier, miR399 has been defined as important in phosphate homeostasis in different species (Jones-Rhoades 2011). It controls the levels of PHO2 mRNA, encoding an E2 ubiquitin conjugase, demonstrating that a microRNA can target a regulator different from a transcription factor and even more interesting, that microRNAs can be transported through the phloem to transmit a stress signal (Pant et al. 2008). These examples clearly show the diversity of functions and regulatory circuits in which microRNAs can be involved. The challenge is to understand other relevant microRNA activities to the extent these cases have shown, in plants different from Arabidopsis and under particular stress conditions. A promising way to start is by analyzing the global microRNA expression profile using genome-wide approaches as described next.

Genome-Wide Analyses

Over the last decade, there has been an explosion of microRNA data due to the advent of genome-scale technologies to explore small RNAs by high-throughput sequencing or microarray analysis. In particular, we have seen that stress-related microRNAs have been characterized for diverse plant models. To name a few recent examples, microRNA deep-sequencing data has emerged for *M. truncatula* in response to aluminum toxicity or drought (Chen et al. 2011a; Wang et al. 2011), drought or cold in two *Populus* species (Chen et al. 2011b; Li et al. 2011a), multiple forms of biotic and abiotic stress in soybean (Kulcheski et al. 2011; Li et al. 2011b),

among others. This methodology has provided valuable information about how microRNAs are involved in stress responses: which microRNAs are expressed in a specific abiotic/biotic stress condition, and the possibility to identify novel microRNAs as well as other regulatory small RNAs. Due to the large scale of the results obtained, estimations of microRNA differential expression under stress conditions can be inferred as well.

As mentioned earlier, high-throughput sequencing can also be applied to explore the population of mRNA molecules that has been processed by small RNA cleavage. This type of analysis, commonly known as 'Degradome' analysis, provides short sequencing reads that match sites at mRNAs that are prone to degradation and that upon comparison to known or predicted small RNAs can reveal mRNAs under microRNA regulation. This approach provides experimental validation to complement bioinformatical predictions, and has been extended to analyze RNA target processing by tasiRNAs, sites of RNA-dependent DNA methylation (RdDM) implicated in chromatin silencing and even microRNA precursor processing. A recent report in soybean used deep sequencing of small RNAs and degradome analysis to report 26 new miRNAs and 9 miRNAs belonging to conserved microRNAs families and defined 170 transcript targets that could have a function during soybean seed development (Song et al. 2011). Using a similar approach 21 novel microRNAs and 112 mRNA targets were described for *Vitis vinifera* (Pantaleo et al. 2010). In terms of stress responses, a recent study combining deep sequencing of small RNAs and cleaved mRNA targets in maize seedlings exposed to nitrogen deficiency expanded the number of known microRNAs by identifying a total of 99 new loci and confirming 108 target mRNAs (Zhao et al. 2012). Furthermore, responses to drought conditions in leaves of *P. euphratica*, a known stress-resistant woody species, were evaluated through the use of genome-wide strategies: small RNA high-throughput sequencing revealed 58 novel microRNAs (in addition to others already known), while degradome analysis confirmed 47 targets for conserved and novel microRNAs (Li et al. 2011a). Interestingly, this study also used microRNA-specific microarrays to compare with results obtained from deep sequencing and to evaluate the accumulation of microRNAs due to stress conditions.

Small RNA microarrays represent an alternative to high-throughput sequencing to explore the accumulation status of microRNAs. Due to the ability to perform replicates more easily than with sequencing strategies, results obtained with microarrays are more amenable to statistical analysis and can include samples from multiple origins (developmental stages, organs, time points, etc.). A disadvantage is that the evaluation of a global microRNA profile is limited to sequences present in the array and prevents the ability for small RNA discovery. Nevertheless it has been recently used to successfully evaluate microRNA profiles in response to cadmium toxicity (Ding et al. 2011) or during the course of drought at two developmental stages in rice (Zhou et al. 2010), to explore shock drought in *Triticum dicoccoides* leaves and roots (Kantar et al. 2011) or to compare two cotton cultivars differing in their susceptibility to salt stress (Yin et al. 2011), to mention a few recent examples that show how a variety of conditions can be assessed using microarrays. A combination of genome-wide technologies has allowed for a

glimpse of the intricate microRNA populations present during stress conditions in plants. The results are now available and will help to determine the contribution of individual microRNAs to plant responses.

Genetic Screens

HYL1 was the first factor involved in the microRNA pathway that was originally isolated as a mutant with a defect in its response to stress. *hyl1* plants are hypersensitive to ABA in addition to other developmental defects, and less sensitivity to auxin and cytokinin (Lu and Fedoroff 2000). Later on, it was recognized that HYL1 participates in precursor processing by aiding DCL1 to correctly recognize cleavage sites together with SE along the precursor secondary structure (Dong et al. 2008). These findings suggested that impairment of microRNA biogenesis might cause a deficiency in a particular microRNA and a consequent disruption of its role in modulating ABA responses. Alternatively, the defect on stress responses could be due to an indirect effect through general failure to accumulate the appropriate amounts of the microRNA population within the cell. In either case, it is expected that mutations in other elements participating in microRNA biogenesis show similar phenotypes.

For example, the *ABA HYPERSENSITIVE 1* (ABH1) gene identified as a mutant in CBP80, the large subunit of the cap binding complex (CBP), showed defects in ABA sensitivity, and reduced wilting upon drought treatment (Hugouvieux et al. 2001). Interestingly, mutations in the CBP20 subunit also turned out to be resistant to drought and ABA hypersensitive during germination (Papp et al. 2004). Because *cbp20* and *cbp80* mutants contained reduced levels of mature microRNA but increased levels of their precursors (Kim et al. 2008), it was postulated that correct recognition of the primary transcript for multiple microRNAs could be impaired in the mutants and in turn result in diminished levels of microRNAs involved in ABA responses, such as miR159 (Reyes and Chua 2007). Another screen recovered *ABA supersensitive during germination* (*absg*) mutants, revealing new alleles for *DCL1* and *HEN1* in Arabidopsis, resulting in hypersensitivity to ABA as well as enhanced sensitivity to drought and salt stress, and increased expression of stress-responsive genes (Zhang et al. 2008). That microRNA biogenesis factors are involved in stress responses is further supported by the finding that the mRNAs encoding *Mt*DCL1 and *Mt*AGO1 increased their accumulation, while microRNAs known to regulate these transcripts, namely miR162 and miR168, decreased their levels in *M. truncatula* roots in response to water deficit (Capitao et al. 2011). Moreover, identification of *DCL* and *AGO* genes in maize and subsequent expression profiling indicated that certain members of these families are affected by osmotic and salt stress (Qian et al. 2011). In contrast to these findings, FBW2 encodes an F-box protein that negatively regulates the levels of Arabidopsis AGO1 protein. The *fbw2* mutant exhibits decreased ABA sensitivity, the opposite phenotype to many microRNA biogenesis mutants, correlating with

increased AGO1 abundance (Earley et al. 2010). These results reinforce the idea that the microRNA regulatory pathway is intimately involved in stress responses, and while many examples of specific microRNAs participating at different levels in stress responses have been identified (section *MicroRNAs Involved in Stress Responses*), it is still uncertain what is the largest effect of mutants in this pathway: the absence of certain microRNAs or impairing the overall microRNA levels. While precise and detailed analyses could address this question it is likely to be a combination of both effects what determines the outcome of plant response to adverse conditions.

Future Perspectives

Much has been learned from the study of microRNA expression in the context of stress affecting major plant models, including Arabidopsis, rice, maize, and crops such as legumes, and others. The current evidence has revealed that during a wide variety of adverse conditions the entire landscape of the microRNA population changes according to the condition imposed. In certain cases these changes result in a large variation in microRNA abundance while others are subtler. This scenario should then be reflected in the abundance of those transcripts regulated by the action of microRNAs and possibly other small regulatory RNA molecules, such as tasiRNAs or small RNAs arising from alternate processing of specific precursors. While a large amount of work has been put into identifying the microRNA contribution to this regulatory circuit, much less has been revealed about their mRNA targets. Future work will benefit from large-scale analysis such as degradome or transcriptome analysis to unveil these targets based on the use of a variety of experimental and bioinformatical tools. A subsequent analysis of the changes in mRNA abundance caused by stress conditions should be aimed at integrating the individual effects of microRNA regulation observed into regulatory networks to reveal the effect on cellular pathways and metabolism and how they are ultimately affecting the plant responses to abiotic stress conditions.

Another factor contributing to the outcome of microRNA regulation is the relative abundance of these and other small RNAs within the cell. As we mentioned above, several factors contribute to the final concentration of the small RNA molecules, including, but not limited to, transcription rate, half-life, competition among target mRNAs for microRNA availability, and possibly others yet to be discovered. The cell machineries responsible for these processes might as well be regulated by stress conditions. We exemplified this aspect by highlighting the susceptibility to stress developed by mutants in a few of the microRNA biogenesis factors (section *Genetic Screens*), however other factors involved in regulating microRNA abundance might turn out to be affected by stress and thus in turn alter, directly or indirectly, the responses to those stimuli that affected their function in the first place.

In addition to conserved microRNAs it will be important to analyze less conserved small RNAs that may play important roles in crop species, potentially regulating processes specific to particular plant species. However as it has been mentioned, identification of targets will be essential to place regulatory pathways in these other plants.

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