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Review Article

MicroRNAs play critical roles during plant development and in response to abiotic stresses

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Abstract

MicroRNAs (miRNAs) have been identified as key molecules in regulatory networks. The fine-tuning role of miRNAs in addition to the regulatory role of transcription factors has shown that molecular events during development are tightly regulated. In addition, several miRNAs play crucial roles in the response to abiotic stress induced by drought, salinity, low temperatures, and metals such as aluminium. Interestingly, several miRNAs have overlapping roles with regard to development, stress responses, and nutrient homeostasis. Moreover, in response to the same abiotic stresses, different expression patterns for some conserved miRNA families among different plant species revealed different metabolic adjustments. The use of deep sequencing technologies for the characterisation of miRNA frequency and the identification of new miRNAs adds complexity to regulatory networks in plants. In this review, we consider the regulatory role of miRNAs in plant development and abiotic stresses, as well as the impact of deep sequencing technologies on the generation of miRNA data.

Keywords: miRNAs, development, abiotic stress, nutrients, deep sequencing.

MicroRNAs, Their Synthesis and Processing

Gene transcription is a key mechanism regulated by transcription factors and also by distinct small RNAs of 21 to 24 nucleotide of length that can act at the transcriptional and post-transcriptional level (Jamalkandi and Masoudi-Nejad, 2009; Voinnet, 2009). In plants, the regulation of gene expression mediated by small RNAs initiates after the generation of double stranded RNAs and/or single strand RNAs that are folded into stem-loop/hairpin structures in the cells. These are recognized by RNase III-like enzymes called Dicer-Like (DCL), processed into small interfering RNAs, and loaded into protein complexes (RISC) to effectuate gene silencing after the recognition of different complementary target RNAs and or DNA. Distinct biochemical pathways generate different classes of small RNAs: short interfering RNAs (siRNAs), piwi-interacting RNAs occurring exclusively in animals (piRNAs), trans-acting siRNAs (TAS), naturally anti-sense siRNAs (NAT) and microRNAs (miRNAs) (Ramachandran and Chen, 2008; Chen, 2009; Jamalkandi and Masoudi-Nejad, 2009; Liu and Paroo, 2010). TAS pathway - RNA Pol II transcribes TAS genes into a TAS precursor, which is recognized by a complementary siRNA and sliced by Argonaute (AGO) proteins into small RNA which serves as a template for RNA Dependent RNA Polymerases (RDR) to make dsRNAs. This siRNA duplex originated by Dicer-Like directs cleavage of the TAS precursor in *cis* or another target mRNAs in *trans*. MicroRNA pathway: a MIR gene is transcribed by RNA Pol II into a precursor pri-microRNA which is stabilized and cleaved by a protein complex composed of DCL and Hyponastic Leaves (HYL) into a pre-microRNA, which is further processed into a mature microRNA. The HUA Enhancer (HEN) methylates the resulting mature microRNA form in the 2'-hydroxy termini of both strands. This methylated mature form is exported to cytoplasm through HASTY protein (HST).

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Once in the cytoplasm, AGO proteins recognize the mature microRNA and direct it to the target gene. Later, the AGO can induce the slicing of mRNA target or repress the translation complex. The other microRNA strand is directed to the exosome and degraded by Small RNA Degrading Nuclease (SDN). Natural Acting Small RNAs (NAT) Pathway: overlapping genes can be transcribed by RNA Pol II, resulting in a NAT precursor complementary to an siRNA, which serves as a template to the RDR proteins. The DCL protein cleaves this double-stranded precursor into dsRNAs, which are exported to the cytosol by HST protein. The NAT-siRNAs loaded into AGO complexes induce mRNA degradation in the same way as for the microRNA pathway (Figure 1) (Voinnet, 2009; Krol *et al.*, 2010).

Although there are three major classes of small RNAs, miRNAs have been widely characterised in numerous biological conditions in plants. MiRNA genes originated from inverted duplications and random sequences in the genome (Felippes *et al.*, 2008; Voinnet, 2009). They are transcribed by RNA Pol II into long primary polyadenylated RNA molecules and processed into mature miRNAs by Dicer-Like proteins (Parizotto *et al.*, 2004). In plants, several biological experiments indicate that miRNAs play key roles during development and in response to environmental stresses (Figure 2) (Sunkar, 2010). The growing number of miRNAs has revealed the high complexity of genomes and biochemical and metabolic pathways in plants. Different miRNAs can act as regulators, from very early developmental phases to the reproductive phase (Chen, 2009). Although the study of the regulatory roles of miRNAs uncovered a new field in plant biology, the roles of several miRNAs remain to be discovered.

Here, in a concise review, the regulatory action of miRNAs in development and response to abiotic stress will be discussed. Briefly, the new sequencing technologies will also be addressed, as far as they apply to the characterisation and identification of new miRNAs.

miRNAs and Development in Plants

In plants, mutations in the genes involved in biogenesis and the regulatory roles of miRNAs produce strong effects on development. These effects demonstrate the crucial role of miRNAs in development (Ramachandran and Chen, 2008; Chen, 2009; Xie *et al.*, 2010). The Argonaute genes (*AGO*), especially the miR168a and miR168b-regulated *AGO1*, have a fundamental role in the stabilisation and regulatory action of other miRNAs (Vaucheret *et al.*, 2004). In *Arabidopsis thaliana*, due to overlapping functions among different members of the MIR168 family, mutations in the MIR168a gene did not affect plant development under normal growth conditions. (Vaucheret, 2009). Although some miRNA families are numerous, there are few examples in the literature that uncover functions for individual members in plants (Chen, 2009). One good example is the TF (transcription factors) coding genes No Apical Meristem (*NAM-NAC*) and cup-shaped cotyledon (*CUC*) that are regulated by the miR164 family in *A. thaliana*, which are important in root and shoot development (Baker *et al.*, 2005; Guo *et al.*, 2005; Nikovics *et al.*, 2006; Sieber *et al.*, 2007; Raman *et al.*, 2008). Triple mutants of *miR164abc* revealed that the genes athMIR164a and athMIR164b partially overlap athMIR164c function during floral development, as the phenotype became more severe in the triple mutant (Sieber *et al.*, 2007). Individual mutants for athMIR164a and athMIR164b result in plants with more roots, which diversify the functional role of the miR164 family (Guo *et al.*, 2005). To allow for proper root development, the short root (SHR) and scarecrow like (SCR) proteins activate the MIRNA165a and MIR166b genes, which in turn negatively regulate the TF HD-ZipIII (Carlsbecker *et al.*, 2010). The *osaxr* mutant rice plant insensitive for auxin revealed numerous miRNAs and complex regulatory signals involved in root development (Meng *et al.*, 2009).

It is already known that miR156 regulates the squamosa promoter binding- like (*SPL*) genes and that plants overexpressing miR156 are semi-dwarf, have altered numbers of leaves, and have longer vegetative phase (Xie *et al.*, 2006; Wang *et al.*, 2008; Zhang *et al.*, 2011b). The down-regulated expression of miR156 from the juvenile to the adult phase is in contrast to the up-regulation of miR172, which is an important regulator of the floral patterning genes such as *APETALA2*, *TOE1* and *TOE2* (Aukerman and Sakai, 2003; Wu *et al.*, 2009; Zhu and Helliwell, 2010). Interestingly, the dominant corngrass (*Cg1*) mutant, which contains two tandem MIR156 genes, showed an over-expression of miR156 in the meristem and lateral organs, and reduced levels of miR172, suggesting that the regulatory roles for these two miRNAs in the transition from the juvenile to the reproductive phase in maize and other plants is conserved (Chuck *et al.*, 2007). Recently, ablation of leaf primordia delayed the transition to the reproductive phase, revealing that transcriptional signals that modulate miR156 regulatory action were found to be crucial for phase change in plants (Yang *et al.*, 2011). In maize, it was determined that the spatial expression of miR156, which regulates the TF *ZmTSH4*, is crucial for the establishment of the lateral meristems (Chuck *et al.*, 2010). Strikingly, the posttranscriptional regulation of *OsSPL14* by miR156 defines the rice plant architecture, with mutants displaying a reduced number of tillers and more branches in the panicles (Jiao *et al.*, 2010; Miura *et al.*, 2010). Throughout the different developmental stages of soybean seeds, 26 new miRNAs and their target genes were identified using deep sequencing and degradome approaches (Song *et al.*, 2011).

Hormone signalling and gene expression under miRNA control have deterministic roles in plant development (Liu and Chen, 2009; Liu *et al.*, 2009). In *A. thaliana*,

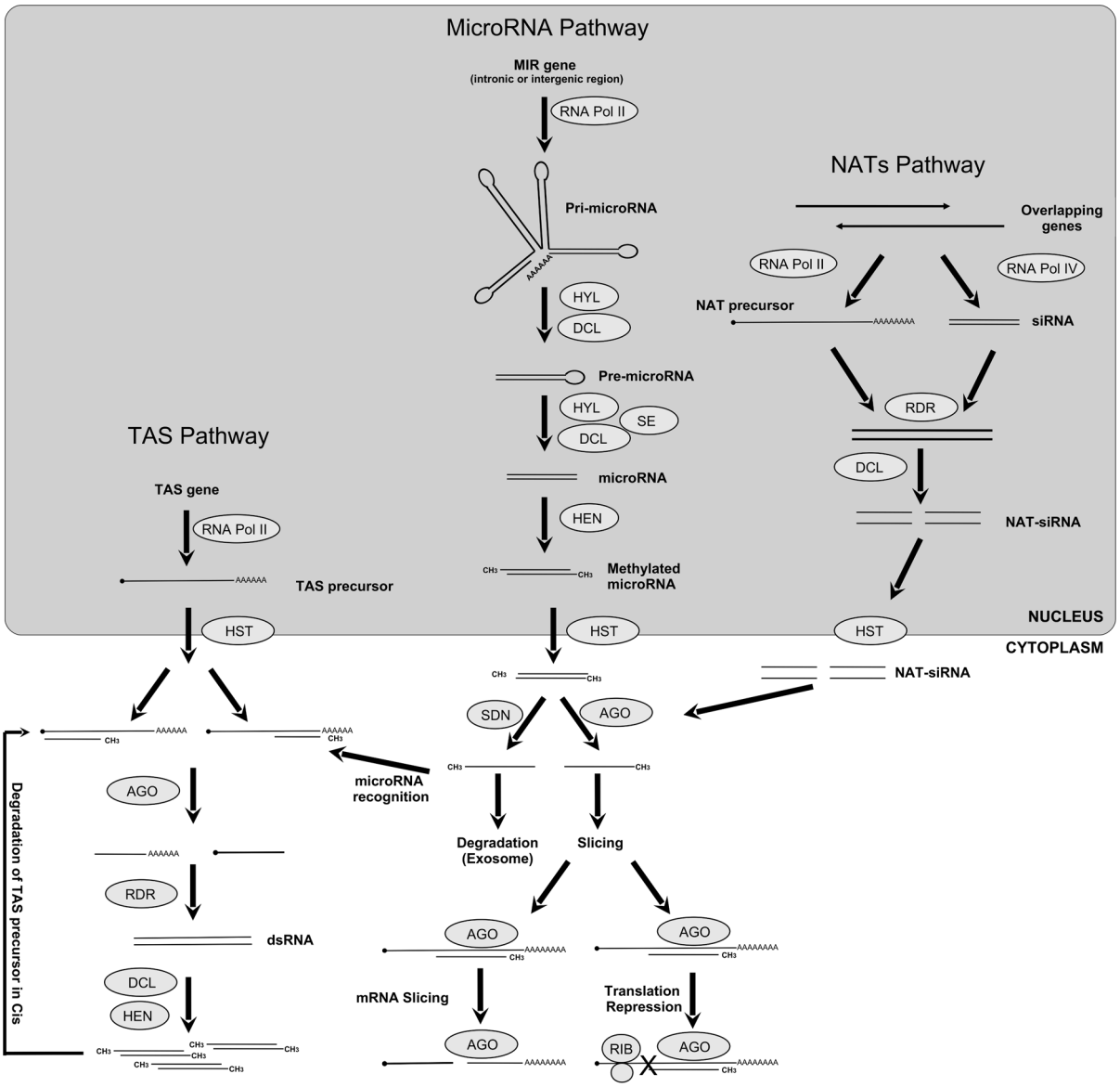

Figure 1 - General view of the small RNA pathways in plants. Cis/Trans-Acting small interfering RNAs (TAS) Pathway: A TAS gene is transcribed by RNA Pol II into a TAS precursor, later this precursor is recognized by a complementary siRNA and sliced by Argonaute (AGO) proteins into small RNA which serves as a template for RNA Dependent RNA Polymerases (RDR) to make dsRNAs. This siRNA duplex originated by Dicer-Like directs cleavage of the TAS precursor in *cis*, or another target mRNAs in *trans*. MicroRNA Pathway: A MIR gene, usually located in intergenic or intronic region, is transcribed by RNA Pol II into a precursor RNA named pri-microRNA, which is stabilized and cleaved by a protein complex composed by Dicer-like proteins (DCL) and Hyponastic Leaves (HYL) into a pre-microRNA which is further processed by the same complex plus Serrate (SE) into a mature microRNA. The HUA Enhancer (HEN) methylates the resulting mature microRNA form in the 2'-hydroxy termini of both strands. This methylated mature form is exported to cytoplasm through HASTY protein (HST). Once in the cytoplasm, AGO proteins recognize one strand of the mature microRNA and direct it to the target gene. Later, the AGO can induce the slicing of mRNA target or repress the translation complex. The other microRNA strand is directed to the Exosome and degraded by Small RNA Degrading Nuclease (SDN). Natural Acting Small RNAs (NAT) Pathway: Overlapping genes can be transcribed by RNA Pol II, resulting in a NAT precursor complementary to an siRNA, which serves as a template to the RDR proteins. The DCL protein cleaves this double-stranded precursor into dsRNAs, which are exported to the cytosol by HST protein. The NAT-siRNAs loaded into AGO complexes induce mRNA degradation in the same way as for microRNA pathway.

miR159-targeting members of the gibberelic acid MYB (GAMYB) family regulates seed germination and anther formation (Reyes and Chua, 2007). The overexpression of miR159 and the inhibition of the *MYB* gene expression delayed flowering and caused male sterility (Millar and Gubler, 2005). In Arabidopsis plants, *miR159ab* deregulation of the *GAMYB*-like genes resulted in reduction of the cell proliferation and programmed cell death (Alonso-Peral *et al.*, 2010). Jasmonic acid biosynthesis is regulated by the teosinte branched/cycloidea proteins (TCP), which are TFs. These TFs have functional roles in development and leaf senescence and are regulated by miR319 (Schommer *et al.*,

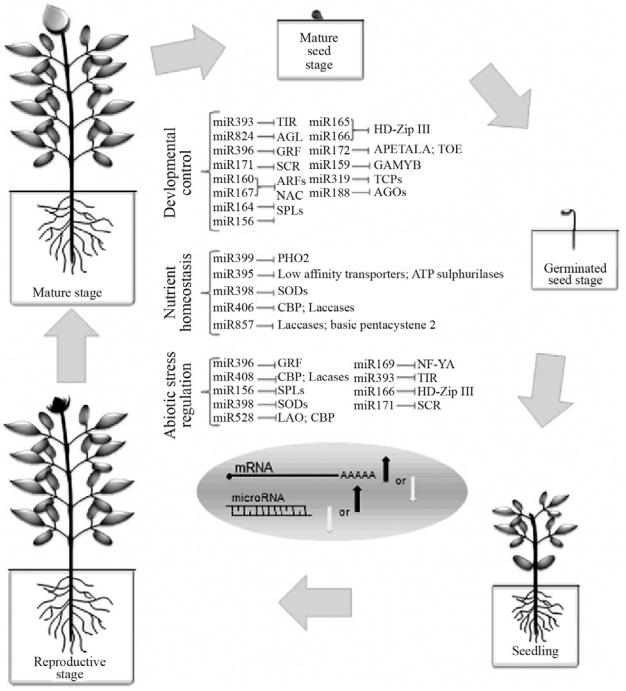

Figure 2 - Mature miRNAs act in plant development, in response to abiotic stresses, and also in the control of nutrient homeostasis. Depending on the plant species, the miRNA/target genes are modulated differently in the same biological conditions. Only miRNA targets already confirmed by expression analysis and/or degradome sequencing were included in this diagram. TIR: F-box protein; AGL: agamous like; GRF: growth regulating factor; SCR: scarecrow like; ARF: auxin response factor; NAC (NAM): no apical meristem; SPL; squamosa promter binding like; HD-Zip III; homeodomain transcription factor; APETALA; GAMyb: Gibberelic Acid Myb transcription factor; TCPs: Teosinte Branched/cycloidea transcription factor; AGOs: Argonaute; PHO2: phosphate 2 - E2 Conjugase protein; Low affinity transporter; ATP sulphurilases; SODs: superoxide dismutase; CBP: Copper Ion Binding Protein; Laccases; Basic pentacysteine 2; L-AO: L- ascorbate oxidase; NF-YA: CCAAT-box binding transcription factor.

2008). Functional genes in the auxin signalling pathway (ARFs – auxin response factors) are miRNA targets. Plants with miR160-resistant forms of the *ARF10*, *ARF16* and *ARF17* genes showed pleiotropic effects in shoots and roots (Mallory *et al.*, 2005; Liu *et al.*, 2007). The overexpression of miR160 resulted in plants with less sensitivity to giberelic acid during germination (Liu *et al.*, 2007). In flowering, during stamens and gynoecium development, miR167 has an important role when targeting *ARF6* and *ARF8* genes (Wu *et al.*, 2006). The SCR family is targeted by miR171c to promote the proper development of auxiliary meristems during branching (Wang *et al.*, 2010). Cell proliferation in *A. thaliana* is attenuated by the upregulation of miR396, which downregulates the growth regulating factor genes (*GRF*) that are crucial regulators in the cell cycle (Rodriguez *et al.*, 2010). The occurrence of stomata, crucial for plant transpiration, depends partially on miR824 targeting the agamous like 16 gene (*AGL16*) (Kutter *et al.*, 2007).

miRNAs in Response to Abiotic Stresses

In addition to the role of miRNAs in plant development, they are dramatically affected under abiotic stresses, where they regulate several coding genes in plants (Reyes *et al.*, 2010; Sunkar, 2010). An understanding of how miRNAs act when they regulate gene expression and which coding genes are miRNA targets during stress responses, such as drought, salinity, metals, temperature and nutrient homeostasis, will help the generation of more tolerant plants (Sunkar, 2010).

The Regulatory Role of miRNAs in Plants Under Drought, Salinity, Aluminum, and Low Temperatures

It has been suggested that miR393 is one of the key miRNAs during stress responses because of its altered expression in *A. thaliana*, *Oryza sativa*, *Medicago truncatula*, *Phaseolus vulgaris* and other plants under drought, salinity, low temperature, and aluminium stress conditions (Sunkar and Zhu, 2004; Zhao *et al.*, 2007; Liu *et al.*, 2008; Arenas-Huertero *et al.*, 2009; Trindade *et al.*, 2010). However, the molecular evidence that miR393 regulates its targets in several environmental conditions remains to be considered. Recently, *Arabidopsis* plants overexpressing osaMIR393 became more tolerant to salt excess, suggesting a regulatory role in salinity tolerance (Gao *et al.*, 2011). It is known that miRNAs from the miR169 family respond differently to drought, salinity, low temperatures and aluminium in plants (Zhao *et al.*, 2007; Liu *et al.*, 2008; Zhou *et al.*, 2008; Zhao *et al.*, 2009). In response to salinity and drought stress in rice, the expression of the nuclear transcription factor YA (*NF-YA*) genes is modulated by members of the miR169 family (Zhao *et al.*, 2009). In *A. thaliana*, *nf-ya* plants and plants overexpressing miR169 are more sensitive to drought (Li *et al.*, 2008). On the contrary in tomato, plants overexpressing miR169c, which targets a gene involved in the opening and closing of stomata, are more tolerant to drought (Zhang *et al.*, 2011a). A reduction in the expression of miR530a, miR1445, miR1446a-e and miR1447 in *Populus trichocarpa* was detected in plants under drought and salinity, which is different from the miR1450 pattern of expression, downregulation under drought conditions and upregulation under high salinity (Lu *et al.*, 2008). In *Triticum dicoccoides*, the ancestor of cultivated wheat, the upregulation of miR1450 revealed an inverse response when compared with *Populus trichocarpa* under drought conditions (Kantar *et al.*, 2011). Although the gene MIR1450 is present in both monocot and dicots, the expression patterns suggest regulatory differences under drought (Lu *et al.*, 2008; Kantar *et al.*, 2011). The formation of the superoxide anion O^{2-} in response to stresses is converted into less toxic molecules by superoxide dismutases SOD1 and SOD2 proteins, whose mRNAs are targeted by miR398 (Sunkar *et al.*, 2006; Jagadeeswaran *et*

al., 2009; Trindade *et al.*, 2010; Kantar *et al.*, 2011). The inverse correlation between miRNAs miR156, miR166, miR171, miR408 and their targets were detected in barley plants under drought (Kantar *et al.*, 2010). In different tissues from different developmental stages in rice plants under drought conditions in soil, miRNAs miR156, miR171 and miR408 were also detected (Zhou *et al.*, 2010). In *Medicago truncatula*, miR408 acts to regulate plantacyanin genes in response to drought (Trindade *et al.*, 2010). In acidic soils, the availability of aluminium in low pH conditions inhibits root growth, which affects plant development dramatically (Ryan *et al.*, 2011). Comparing *japonica* and *indica* subspecies, which differ in aluminium tolerance, we have characterised the expression of miRNAs in rice plants treated with aluminium. Using RT-qPCR, it was possible to detect sixteen differentially expressed miRNAs in rice roots, which reveals a complex miRNA response in rice under aluminium stress. The inverse regulation of miR528 and its targets L-ascorbate oxidase (L-AO) and copper ion binding protein genes was also observed. This finding corresponds to the first report on the characterisation of the miRNA response in plants under aluminium stress (Lima *et al.*, 2011).

miRNAs and Their Regulatory Role in the Response to UV-B Radiation, Hypoxia, and Oxidative Stress

The redox state of the cellular environment and the generation of ROS as a consequence of UV-B radiation and hypoxia reprograms plant responses due to eminent irreversible damage (Blokhina and Fagerstedt, 2010; De Gara *et al.*, 2010). The induction of different microRNAs in maize plants under low oxygen points to a diverse role of miRNAs in morphological and physiological adaptations in root cells and in sulphur and oxidative metabolism (Zhang *et al.*, 2008). The repression of miR398 and the upregulation of SOD proteins has a crucial role in *Arabidopsis* plants under oxidative stress (Sunkar *et al.*, 2006). The downregulation of miR395 and the induction of miR398, as well as the respective inversion of expression of their targets in response to UV-B in *Populus tremula*, suggests there are important differences in the stress-induced metabolic adjustments compared with *Arabidopsis* (Jia *et al.*, 2009). By deep sequencing, the identification of miRNAs in rice plants under hydrogen peroxide treatment has broadened the roles for miRNAs in plants under oxidative stress**.** Targets of these hydrogen peroxide-responsive miRNAs are involved in different cellular responses and metabolic processes including transcriptional regulation, nutrient transport, auxin homeostasis, cell proliferation and programmed cell death, which indicates the diversity of miRNAs function in plants' responses under oxidative stress (Li *et al.*, 2010a).

The Importance of miRNAs in Nutrient Homeostasis

The uptake of nutrients is a compulsory requirement of plants, and the homeostasis of nutrients is critical for the maintenance of growth and development (Giehl *et al.*, 2009; Yang and Finnegan, 2010). Sulphur is transported into the cell as sulphate and has a structural role in protein folding (Rausch and Wachter, 2005). Under sulphate deficiency, miR395 down-regulates low affinity transporters and ATP sulphurilases (Jones-Rhoades and Bartel, 2004). Interestingly, in *Arabidopsis* roots, both the sulphate transporter AST68 and miR395 were induced. The spatial expression patterns suggested that miR395 limits the expression of its targets in the phloem cells (Kawashima *et al.*, 2009). Phosphate (Pi) homeostasis is under miR399 regulation. Under low cellular phosphate levels, Pi responsive gene (PHR1) activates miR399, which negatively regulates the phosphate 2 gene (*PHO2*), which has a role in protein degradation pathways (Bari *et al.*, 2006). An alternative regulation in phosphate signalling is the expression of a non-coding RNA called IPS (Induced by Phosphate Starvation), which has a miR399 binding site with some mismatches that impair IPS cleavage by miR399 under ideal phosphate conditions. The sequestration of miRNAs by IPS (target mimicry) blocks the down-regulation of PHO2 by miR399 (Franco-Zorrilla *et al.*, 2007). Although target mimicry needs further investigation in other plants, it is functional and widespread in *Arabidopsis* (Todesco *et al.*, 2010). In addition, by deep sequencing, the detection of several miRNAs revealed a much more complex regulatory network in phosphate signalling (Hsieh *et al.*, 2009; Gu *et al.*, 2010).

Metals like copper and iron are also essential micronutrients to plants. MiRNAs miR398, miR408 and miR857 are part of a signalling network that functions in the regulation of copper levels in plant cells. Under copper deficiency, these miRNAs were induced and negatively regulated their targets (Yamasaki *et al.*, 2007; Burkhead *et al.*, 2009). Several miRNAs were up-regulated in response to low iron levels in *Arabidopsis* (Kong and Yang, 2010). Interestingly, a member of the miR854 family that is induced in plants under iron deficiency conditions is also conserved in animals, and its targets can be regulated via translation inhibition (Arteaga-Vazquez *et al.*, 2006; Kong and Yang, 2010). In nutrient metabolism, miR169 regulates its target *NF-YA* genes, which have an important role in the balance of nitrogen in plants (Zhao *et al.*, 2011).

Next Generation Sequencing Technologies: How to Deal with an Increasing Amount of Data?

Deep sequencing technologies are revolutionising molecular biology (Brautigam and Gowik, 2010), lowering the costs of sequencing and increasing throughput by sev-

eral orders of magnitude (Paszkiewicz and Studholme, 2010). A deep sequencing approach was successfully applied for *de novo* sequencing of plant genomes (Imelfort and Edwards, 2009), metagenomics studies in grapevines (Coetzee *et al.*, 2010), sequencing of natural strains in Arabidopsis (Ossowski *et al.*, 2008), RNA sequencing of different tissues from soybean (Severin *et al.*, 2010) and miRNA identification in many organisms. In addition, deep sequencing approaches applied to the characterisation of miRNA frequency and the identification of new miRNAs that regulate development and abiotic stress responses brought more complexity to regulatory networks in plants (Li *et al.*, 2010a; Song *et al.*, 2011). The growing number of miRNAs identified mainly by deep sequencing is increasing with sequence data in databases as miRBase (Kozomara and Griffiths-Jones, 2010) and the Plant MicroRNA Database (PMRD) (Zhang *et al.*, 2010), a specific databank for plant miRNAs. This database allows for the retrieval of a target gene, promoter sequence, and expression profile for some miRNA genes. However, the rapid increase in molecular data in databases represents only the tip of the iceberg, and these data demand more laborious analyses for the identification of miRNA function.

Concluding Remarks and Future Perspectives

The regulatory role of miRNAs in plants is definitely a subject that will require much more investigation in plant biology. As presented in this review, several miRNAs have been determined to be involved in plant development and abiotic stress responses. They are positioned for the fine-tuning of distinct regulatory networks. In addition, the identification of new miRNAs and their targets adds more complexity to gene expression regulatory networks. The increasing number of miRNAs identified by deep sequencing, in different and multiple experimental conditions, points to a need for further biological investigation. To address the question raised in the previous section we consider that the latest strategies for the understanding of individual miRNA function as miRNA target mimicry (Franco-Zorrilla *et al.*, 2007), identification of miRNA target genes by degradome approach (German *et al.*, 2008; Li *et al.*, 2010b), and silencing of miRNAs (Eamens and Wang, 2011) are suitable molecular tools to unravel the functional role of the increasing number of miRNAs (Figure 3). Finally, since miRNAs regulate numerous transcription factors during development and in response to different stresses, high throughput analysis as RNA-seq (deep sequencing of mRNAs), proteomics and metabolomics should be always considered as complementary approaches to investigate the global effects of the conservation and diversity of miRNA responses in different biological conditions and in different plant species.

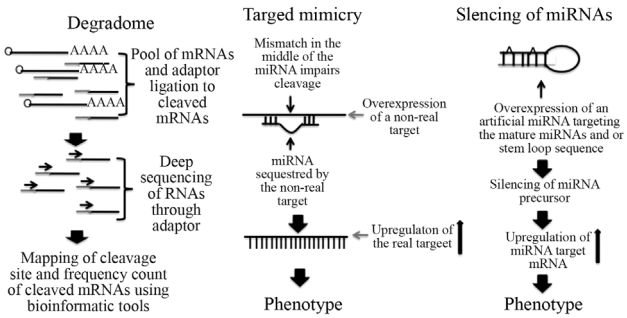

Figure 3 - General representation of the degradome sequencing approach, miRNA target mimicry, and silencing of miRNAs. The degradome sequencing approach has been used to detect miRNA target genes through deep sequencing of cleaved mRNAs and mapping of cleaved sites. Target mimicry is a suitable molecular tool that is based on the expression of a transgene carrying a non-real target of a miRNA that is partially complementary to the miRNA. This RNA contains three to four nucleotides that are not complementary to a core region in the middle of the miRNA, which causes the sequestration of miRNAs, blocks cleavage and up-regulates the real target mRNAs. Silencing of miRNAs triggered by artificial miRNAs targeting the mature and or precursor miRNA, directs cleavage and RNA silencing of the precursor miRNAs and upregulation of miRNA target RNAs.

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