

The Role of MiRNAs in Auxin Signaling and Regulation During Plant Development

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Abstract Auxins are involved in almost every aspect of plant physiology. For instance, auxins play a central role in the differentiation process during the development of plants. Furthermore, the homeostasis of auxins involves biosynthesis and degradation as well as their conjugation with amino acids and carbohydrates, and the hydrolysis of some of these conjugates liberates indole-3-acetic acid (IAA). The balance in the IAA concentration triggers its own signal transduction pathway and produces a molecular and biochemical response. This response begins with the sensing of the IAA concentration through the construction of a co-receptor complex that includes an F-box protein from the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN SIGNALING F-BOX PROTEIN (AFB) family and a member of the AUXIN/IAA-INDUCIBLE (AUX/IAA) family of transcriptional repressors. This complex allows the expression of auxin response genes. Most of the auxin-regulated processes are tightly regulated. Several differentially expressed miRNAs, which alter the auxin response, have been identified in *Arabidopsis thaliana* somatic embryogenesis development. Also, during the stress response in soybean roots, auxin-responsive *cis*-elements in the promoters of many salt-responsive miRNAs have been found. These findings suggest that miRNAs may be regulated by auxins. In this chapter, we analyze developing research related to the interaction between auxins and miRNAs.

Keywords Auxins • Development • miRNAs

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1 Introduction

1.1 Auxins

As sessile organisms, plants have sophisticated development mechanisms to overcome challenges of growth in a hostile environment. Central to this operation are the substances known as plant growth regulators (PGRs), which can trigger multiple responses to influence specific physiological responses. PGRs include cytokinins, ethylene, abscisic acid, gibberellins, auxins, and others and are involved in all phases of plant development, from the response and adaption to recurring biotic and abiotic stresses to seed-to-seed signaling (Weijers et al. 2006; Zhu and Lee 2015). Among PGRs, auxins were the first to be isolated and are probably the most studied. Early studies on the phototropic curvature of coleoptiles by Charles Darwin and his son Francis suggested the existence of a mobile signal that controls cell elongation; this signal was later named auxin and identified as indole-3-acetic acid (IAA) [reviewed in Su et al. (2015)]. Subsequent studies have also demonstrated that auxins are involved in many biological processes, such as embryogenesis, organogenesis, vascular tissue differentiation, hypocotyl and root elongation and apical dominance, among other important processes during the development of plants (Berleth et al. 2000; Leyser 2005; Woodward and Bartel 2005; Benjamins and Scheres 2008; Robert and Friml 2009; Zhao 2010; Ayil-Gutiérrez et al. 2013; Pacurar et al. 2014).

Further studies determined that IAA is mainly synthesized in young leaves, cotyledons, expanding leaves, and root tissues (Ljung et al. 2001; Ljung 2013) by two major pathways: the Trp-dependent and the Trp-independent (Zazimalová and Napier 2003; Woodward and Bartel 2005; Normanly 2010). The IAA biosynthesis pathways are highly conserved throughout the plant kingdom, and four Trp-dependent pathways have been described, including 3-acetaldoximine (IAOx), indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM), and tryptamine (TAM). The conversion of Trp to indole-3-pyruvate and subsequently into IAA by the TRYPTOPHAN AMINOTRANSFERASE and the YUCCA (YUC) family of

flavin monooxygenases, respectively, is the predominant Trp-dependent pathway (Zhao et al. 2001; Stepanova et al. 2008; Tao et al. 2008; Mano and Nemoto 2012). In contrast, evidence exists for a Trp-independent pathway that involves the conversion of indole-3-glycerol phosphate to IAA (Strader and Bartel 2011; Nonhebel 2015; Wang et al. 2015).

Once auxins are synthesized, they have to be differentially distributed throughout the different tissues of the plant. Auxins can move through the phloem or via the cell-to-cell transport system (Habets and Offringa 2014). This distribution is required for local auxin accumulation and the generation of gradients during crucial stages of growth and development (Geisler et al. 2014; Soriano et al. 2014). It has been shown that IAA can move through the plasma membrane by passive diffusion in its uncharged form (IAAH), whereas in its anionic form (IAA⁻) it requires specific auxin efflux and influx carriers (Petrášek and Friml 2009). Efflux carriers such as AUX1 and its related LIKE AUX1 (AUX/LAX) proteins seem to be crucial, especially when the auxin efflux is high, although PIN-FORMED (PIN) proteins, which also act as efflux carriers, contribute to polar distribution and high directionality of auxins according to growth responses (Friml 2003; Petrášek and Friml 2009). Another family group of proteins known as ATP-binding cassette subgroup B can act as efflux and influx carriers. In this way, development in plant cells largely depends on auxin accumulation in the right place at the right moment (Friml et al. 2003). IAA levels are tightly regulated through its conjugation with amino acids or carbohydrates, and the hydrolysis of some of these conjugates liberates IAA according to internal cellular requirements. Therefore, IAA perception inside the cell must be able to change transcriptional events before it produces a biological response.

The regulation of auxin response genes are principally mediated by two families of transcription factors, the auxin response factor (ARF) and the auxin/indole-3-acetic acid (AUX/IAA) (Guilfoyle 2015). At low levels of auxin, the AUX/IAA proteins interact with ARF family proteins, which are targeted to auxin response promoter elements (AuxRES) in several auxin-regulated genes. Thus, to repress the ARF function, these are kept away from their target promoter by AUX/IAA proteins. Additionally, the effects of auxin on transcription also involve changes in chromatin structure and histone modifications. For instance, TOPLESS (TPL) and TPL-related proteins (TRP), other transcriptional repressors, downregulate gene expression through diverse transcriptional regulators. It has been shown that TPL/TRP can recruit histone deacetylases to promote heterochromatin generation, and thus block the ARF function [reviewed in Perrot-Rechenmann (2014) and Retzer et al. (2014)].

In contrast, when the auxin level increases, the auxin binds to TIR1, a component of the SCF (SKP1, CUL1, and F-box protein) ubiquitin ligase complex. This complex promotes AUX/IAA degradation using the 26S proteasome (Gray et al. 1999; Jing et al. 2015). Control of AUX/IAA protein degradation is important for activating the plant cells' response to auxin. Due to AUX/IAA forming heterodimers with ARF, auxin-induced degradation of AUX/IAA reactivates the

ARF protein's function, and thus activates the transcription of primary genes (Weijers 2015; Dinesh et al. 2016).

Independently of the TIR1 auxin receptor, it is also proposed that the perception of extracellular auxin is mediated by auxin-binding protein 1 (ABP1), through interaction with a transmembrane kinase (TMK). ABP1 and TMK form a cell surface auxin perception complex, where the auxin that activates the Rho-like guanosine triphosphatases (ROP) signaling pathway regulates a plethora of plasma membrane or cytoplasmic responses more than it regulates transcriptional activity (Xu et al. 2014).

Taken together, auxin controls most, if not all, aspects of plant growth and development. Although key genes involved in biosynthesis, transport, response, and degradation have been identified, it is still not known how the different levels of this molecular signal are individually perceived by plant cells to generate a molecular or physiological response.

1.2 *MiRNAs*

Ribonucleic acid (RNA) is a wonderful macromolecule, performing a set of essential functions in living organisms. Most genes use mRNA as an intermediate for protein production. However, there are genes whose final products are RNA that do not code for protein. Such non-protein-coding RNAs (ncRNA) range from the transfer and ribosomal RNAs, which are involved in protein-synthesizing machinery, to the more recently discovered regulatory small RNAs (sRNAs). There are several kinds of sRNAs that can be classified into three categories: microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs). There are also new types of sRNAs under investigation, such as small temporal RNAs (stRNAs), small modular RNAs (smRNAs), tiny non-coding RNAs (tncRNAs), trans-acting siRNAs (Ta-siRNAs), repeat-associated siRNAs (Ra-siRNAs), and natural-antisense transcript-derived siRNAs (Nat-siRNAs) (Boopathi 2015).

Mature plant miRNAs are small (20–24 bp in length), and they are produced from longer RNA precursors, which contain a stem loop or hairpin structure with imperfect base pairing in the stem region. MiRNAs are able to regulate gene expression at the post-transcriptional level through specific base pairing with cognate target mRNAs. The recognition of the miRNA by its targeted mRNA produces a cleavage, translation inhibition, or both in the mRNA. The vascular tissue of the plants is the medium through which the miRNAs can move from one tissue to another (Sun 2012).

Since the first reported miRNAs in plants (Llave et al. 2002; Mette et al. 2002; Park et al. 2002; Reinhart et al. 2002), the techniques of massive sequencing and the improvement of the bioinformatics programs have increased the numbers of entries in the microRNA database (<http://www.mirbase.org>; release 21; verified on 28th September, 2016). There are a little more than 7000 miRNAs from plants belonging

Table 1 Presence of miRNAs in plants

	microRNAs	
	Precursors	Mature
Chlorophyta	50	86
Coniferophyta	108	110
Magnoliophyta	124	129
Araliaceae	29	32
Asteraceae	84	94
Brassicaceae	726	1071
Caricaceae	79	81
Cucurbitaceae	120	120
Euphorbiaceae	247	247
Fabaceae	1379	1545
Lamiales	65	71
Linaceae	124	124
Malvaceae	458	460
Ranunculaceae	45	45
Rhizophoraceae	8	8
Rosaceae	386	421
Rutaceae	75	79
Salicaceae	356	405
Solanaceae	463	617
Vitaceae	163	186
Monocotyledons	1616	2221
Total	6992	8496

to 73 species (Table 1), which represents 32% of the total entries in the database. An important segment of the total number of miRNAs come from economically important crops and is highly conserved. MiRNAs participate in the gene regulation of several developmental processes in plants. Among these processes are the response to external environmental stimuli, organogenesis, plant immunity, plant-pathogen interactions, cell proliferation, signaling, and cell death (Boopathi 2015; Shivaprasad et al. 2012).

Even before the discovery of miRNAs in plants, a link among miRNAs and PGRs was established. The hyponastic leaves (*HYL1-1*) mutant exhibited diminished responses to auxin and cytokinin, and hypersensitivity to abscisic acid (ABA) (Lu and Fedoroff 2000). Since then, there has been increasing evidence for the interaction between PGRs and miRNAs, and many of them target mRNAs implicated in auxin responses (Table 2). Auxins are a central player in the regulation of cell division and differentiation, particularly through the interaction with other PGRs, mainly cytokinins (Zhang et al. 2013; Schaller et al. 2015; Xu et al. 2015). However, this interaction goes further and involves brassinosteroids (Zhou et al. 2013) and gibberellins (Liu et al. 2016). Several of these interactions are mediated by miRNAs. Treatment of *Oryza sativa* with PGRs led to the discovery of 22 conserved miRNAs (Liu et al. 2009). Eleven of these were deregulated by one or more

Table 2 Representative examples of auxins' miRNA targets

Species	microRNA	Target	References
<i>Arabidopsis thaliana</i>	160	ARF10	Qiao et al. (2012)
		ARF10, ARF16, ARF17	Kasschau et al. (2003), Mallory et al. (2005), Rhoades et al. (2002), Wang et al. (2005)
	164	NAC1	Guo et al. (2005b), Rhoades et al. (2002)
	166	ARF6, ARF8	Gutierrez et al. (2009)
	167	ARF8	Kasschau et al. (2003), Park et al. (2002), Rhoades et al. (2002)
		ARF6, ARF8	Rhoades et al. (2002), Su et al. (2016)
		ARF10, ARF17	Sorin et al. (2005)
		ARF10, ARF16, ARF17	Mallory et al. (2005)
	319	SAUR IAA3/SHY2	Koyama et al. (2010)
	390	tasiRNAs ARF3, ARF4	Allen et al. (2005), Marin et al. (2010), Yoon et al. (2010)
<i>Glycine max</i>	393	F-box protein family	Iglesias et al. (2014), Navarro et al. (2006), Si-Ammour et al. (2011), Sunkar and Zhu (2004), Wang et al. (2004), Windels and Vazquez (2011)
	847	Aux/IAA	Wang and Guo (2015)
	10515	SUR1	Kong et al. (2015)
<i>Glycine max</i>	156g/j, 172f, 390e, 399a/b, 1511, 2111b/c/f, Gly03, Gly04, Gly16a/b, Gly20, Gly13		Sun et al. (2016)
<i>Raphanus sativus</i>	160, 161	ARF16, ARF17	Zhai et al. (2016)
	167	ARF8	Zhai et al. (2016)
<i>Solanum lycopersicum</i>	160, 167	ARF6, ARF8, ARF10, ARF16	Liu et al. (2016)
	166	ARF6, ARF8	Fan et al. (2015)
<i>Zingiber officinale</i>	167	ARF	Singh et al. (2016)
<i>Coffea</i> spp.	167	ARF8	Chaves et al. (2015)

(continued)

Table 2 (continued)

Species	microRNA	Target	References
<i>Zea mays</i>	160, 167	ARFs	Shen et al. (2013)
	393	F-box protein family	
<i>Oryza sativa</i>	167	ARF8	Yang et al. (2006)

ARF auxin response factor, *CUC1* cup-shaped cotyledons 1, *IAA3/SHY2* INDOLE-3-ACETIC ACID3/SHORT HYPOCOTYL2, *SAUR* SMALL AUXIN UP RNA, *SURI* SUPERROOT1, *tasiRNAs* TAS3-derived trans-acting short-interfering RNAs

PGR treatments. The expression of miR159 and miR394 is regulated by ethylene, while miR167 and miR413 are regulated by ABA (Liu et al. 2009). For auxins specifically, it has been found that miR164 mediates cleavage of *NAM/ATAF/CUC* (*NAC*) domain-encoding mRNAs, in particular *NAC1*, producing an auxin-mediated induction of adventitious and lateral root formation (Guo et al. 2005b). Several of the auxin response factors (ARF), a key component of the auxin signaling cascade, are regulated by various miRNAs. miR160 targets three *ARF*, in particular *ARF17*, modifying the expression of auxin-inducible *GH3* genes, which encode auxin-conjugating proteins (Mallory et al. 2005). On the other hand, *ARF6* and *ARF8* are targeted by miR167 (Gutierrez et al. 2009). Another miRNA that plays an important role in auxin homeostasis is miR393 (Windels and Vazquez 2011; Eckardt 2012). MiR393 downregulates four auxin receptor family F-box protein (*TAAR*) genes (Si-Ammour et al. 2011; Windels and Vazquez 2011).

2 Biogenesis and Function of MiRNAs in Plants

Genetic screening of plant mutants affected in developmental processes, including PGR signaling, has helped to identify many of the genes involved in miRNA biogenesis (Rubio-Somoza and Weigel 2011; Khraiweh et al. 2012). There are many proteins and enzymes involved in miRNA biogenesis, such as HYL1 (HYPONASTIC LEAVES 1), SE (C2H2 Zn-finger protein SERRATE), DCL1 (RNase III DICER-LIKE 1 enzyme), DDL (RNA-binding protein DAWDLE), HEN1 (HUA ENHANCER 1), HASTY (homolog of exportin 5), and AGO1 (ARGONAUTE1) (Ha and Kim 2014).

DNA-dependent RNA Pol II transcribes miRNAs from the *MIR* genes. miRNAs originate from a hairpin or stem-loop precursor (Bartel 2004; Jones-Rhoades et al. 2006; Cuperus et al. 2011). First, the primary miRNA transcript, also called Pri-miRNA, is cleaved by DCL1 to form the stem-loop intermediate precursor pre-miRNA (Tang et al. 2003). The resulting miRNA duplex is then processed by DCL1, the main function of which is to cleave double strand (ds) pri-miRNA or ds pre-miRNA to produce mature miRNA, together with the protein HYL1 in the nucleus (Bartel 2004). The methyltransferase HEN1 incorporates a methyl group on the 2' OH of the 3' last nucleotide of the mature duplex miRNA. These methylations

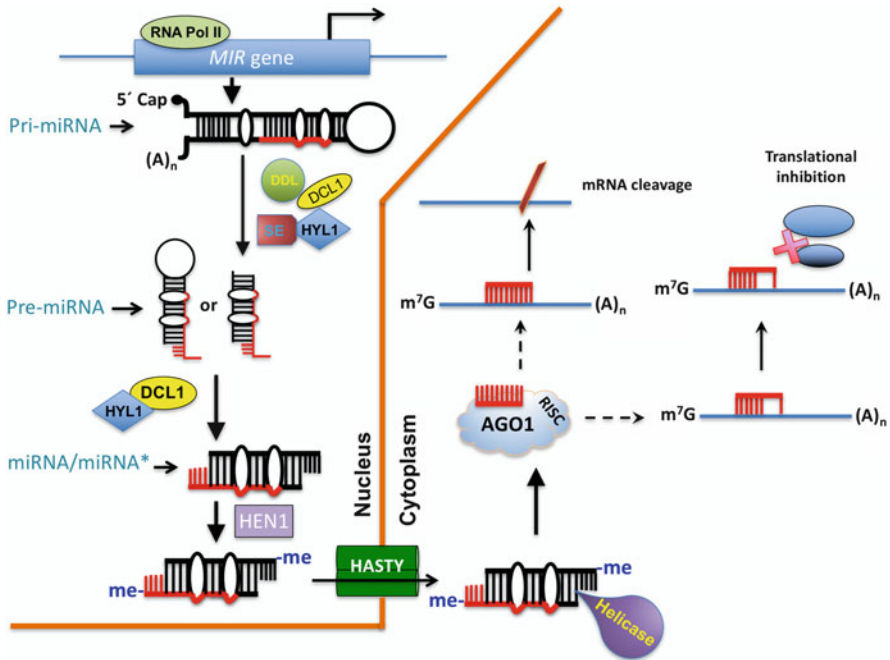


Fig. 1 miRNA biogenesis and silencing mechanisms in plants. MiRNAs are transcribed by DNA-dependent RNA Pol II from the MIR genes. Pri-miRNA is cleaved by RNase III DICER-LIKE 1 enzyme (DCL1). The resulting miRNA duplex is then processed by DCL1. The methyltransferase HUA ENHANCER 1 (HEN1) incorporates a methyl group on the 2'OH of the 3' last nucleotide of the mature duplex miRNA. Then, a homolog of exporting 5 (HASTY) exports the miRNA from the nucleus to the cytoplasm. The mature miRNA is recognized by ARGONAUTE1 (AGO1). There are two types of silencing via miRNAs: one that cleaves the mRNA and one that represses the translation by binding stably to the mRNA targets. Other important players in miRNA biogenesis are DDL (RNA-binding protein DAWDLE), HYL1 (HYPONASTIC LEAVES 1), and SE (C2H2 Zn-finger protein SERRATE). See text for more details

are recognized by HASTY, which exports the miRNA from the nucleus to the cytoplasm. In the cytoplasm, the methyl groups are removed, and a helicase unwinds the ds to produce a single-strand mature miRNA, which is recognized by AGO1 (Bartel 2004). AGO1 is responsible for recruiting all the parts of the RNA-induced silencing complex (RISC) that recognizes the mRNA targets where the mature miRNA has its regulating function (Bartel 2004; Chen 2009). Furthermore, AGO1 is involved in the translational repression of the target mRNAs in the endoplasmic reticulum with the association of AMP1 (ALTERED MERISTEM PROGRAM1) (Li et al. 2013; Iwakawa and Tomari 2013). The resulting biogenesis can generate two types of miRNAs: those which perfectly complement their mRNA targets and those with mismatches to their targets. MiRNAs with perfect complementarity to their target mRNA tend to induce mRNA cleavage by silencing. On the other hand, miRNAs with mismatches tend to repress translation by binding stably to the mRNA targets (Jones-Rhoades et al. 2006; Axtell et al. 2011; Cuperus et al. 2011) (Fig. 1).

The importance of the proteins involved in the miRNA biogenesis during plant development was shown using *A. thaliana* mutants that present phenotypic alterations (Bohmert et al. 1998; Schauer et al. 2002; Mallory and Vaucheret 2006; Chen 2009). For instance, the *dcl1* mutant develops embryo lethality, suggesting that miRNAs are needed for plant viability (Schauer et al. 2002). The *ago1* mutant maintained viability although with dramatic phenotypic changes (Bohmert et al. 1998). An interesting discovery about miRNAs biogenesis is that it can be regulated itself by two miRNAs, miR162 and miR168, targeting *DCL1* and *AGO1*, respectively (Xie et al. 2003; Vaucheret et al. 2004).

3 Evolution of Plant MicroRNA Genes

From the evolutionary point of view, miRNAs can be divided into two groups. In one of the groups are the miRNA families that are highly conserved, from ferns and mosses to higher plants, with only one or two different nucleotides (Llave et al. 2002; Reinhart et al. 2002; Bonnet et al. 2004; Floyd and Bowman 2004; Jones-Rhoades and Bartel 2004; Sunkar and Zhu 2004; Wang et al. 2004; Adai et al. 2005; Axtell and Bartel 2005; Zhang et al. 2005, 2006a, b; Cuperus et al. 2011). In the other group, the non-conserved miRNAs play specific roles in different tissues or plant species (Lu et al. 2005; Zhang et al. 2005; Sanan-Mishra et al. 2009). Certainly, most of the miRNAs present in plants seem to be distinct to that species, and there are many miRNAs that are present only in a base family or are species specific (Axtell 2008, 2013; Axtell and Bowman 2008; Cuperus et al. 2011). Phylogenetic analysis of embryophytes identified eight miRNA families as their common ancestor (Cuperus et al. 2011). On the other hand, the unicellular green alga *Chlamydomonas reinhardtii* does not have any miRNA family in common with the embryophyte plants (Molnár et al. 2007; Zhao et al. 2007). These facts suggest three main hypotheses: (1) that during eukaryotic evolution miRNAs arose at least twice from an ancestral small RNA (Axtell 2008); (2) that individual miRNA families have to be conserved for a long time, remaining basically unchanged since before the appearance of angiosperms (Axtell and Bartel 2005; Axtell 2013); and (3) that most of the known miRNA genes could have arisen relatively recently (Cuperus et al. 2011).

The miRNAs, miR160, miR166, and miR390, involved in response to auxins, are present in all the Embryophyta (Cuperus et al. 2011). Analyzing the miR167 from 20 plant species, we found that all of the miR167-5p do not show any variation among them (Fig. 2). On the other hand, the miR167-3p shows a high level of variability. Half of the species have members in both groups. The other half, including *Brassica rapa*, *B. napus*, *Carica papaya*, *Gossypium hirsutum*, *Nicotiana tabacum*, *Citrus sinensis*, *Vitis vinifera*, *Theobroma cacao*, *Triticum aestivum*, *Sorghum bicolor*, only have members of the conserved family.

An analysis of the presence of miRNAs related to auxins shows that seven of them, 156, 160, 164, 166, 167, 172, and 399, are present in most of the families

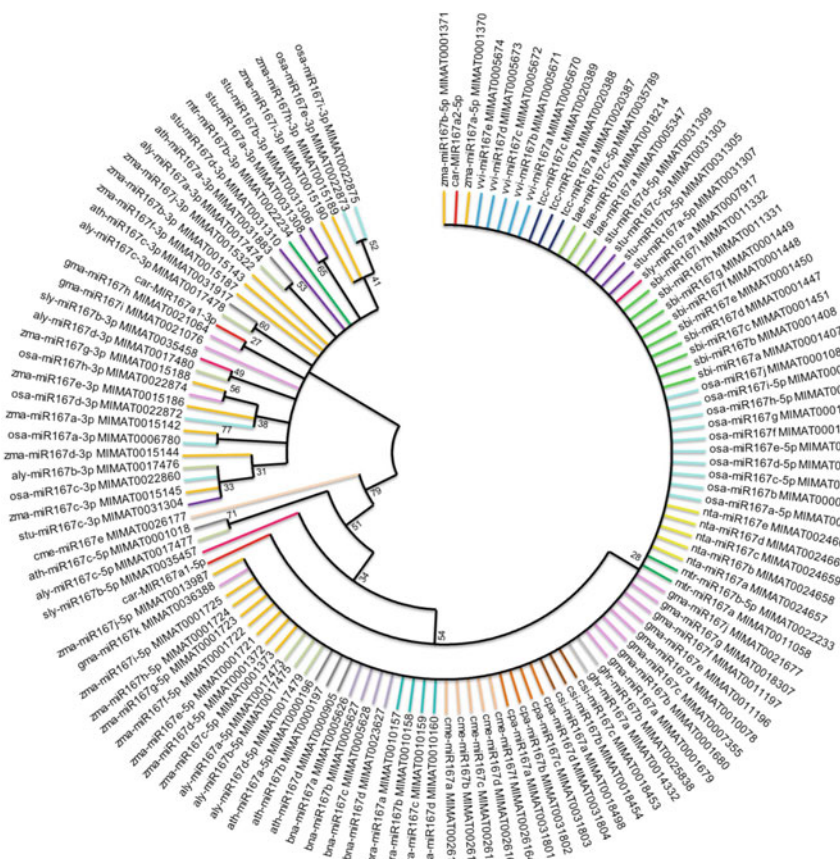


Fig. 2 Phylogenetic relationships of miRNA167 from several species. The tree was constructed using the neighbor joining method using the MEGA program v 6.0. Bootstrap values from 5000 replicates are indicated at each branch. Each color represents one species. Abbreviations: car *Coffea arabica*, osa *Oryza sativa*, ath *Arabidopsis thaliana*, aly *Arabidopsis lyrata*, zma *Zea mays*, cme *Cucumismelo*, cca *Carica papaya*, bna *Brassica napus*, bra *Brassica rapa*, mtr *Medicago truncatula*, nta *Nicotiana tabacum*, sly *Solanum lycopersicum*, stu *Solanum tuberosum*, vvi *Vitis vinifera*, sbi *Sorghum bicolor*, gma *Glycine max*, csi *Citrus sinensis*, tcc *Theobroma cacao*, ghi *Gossypium hirsutum*, tae *Triticum aestivum*

studied (Fig. 3). On the other hand, miRNA161 is present only in the Panicoideae and Brassicaceae, while miR2111 is present in only three families: Fabaceae, Vitaceae, and Brassicaceae. This suggests a clear division among highly conserved miRNAs that are shared among most of the plant families, and very specific miRNAs present in only a few families.

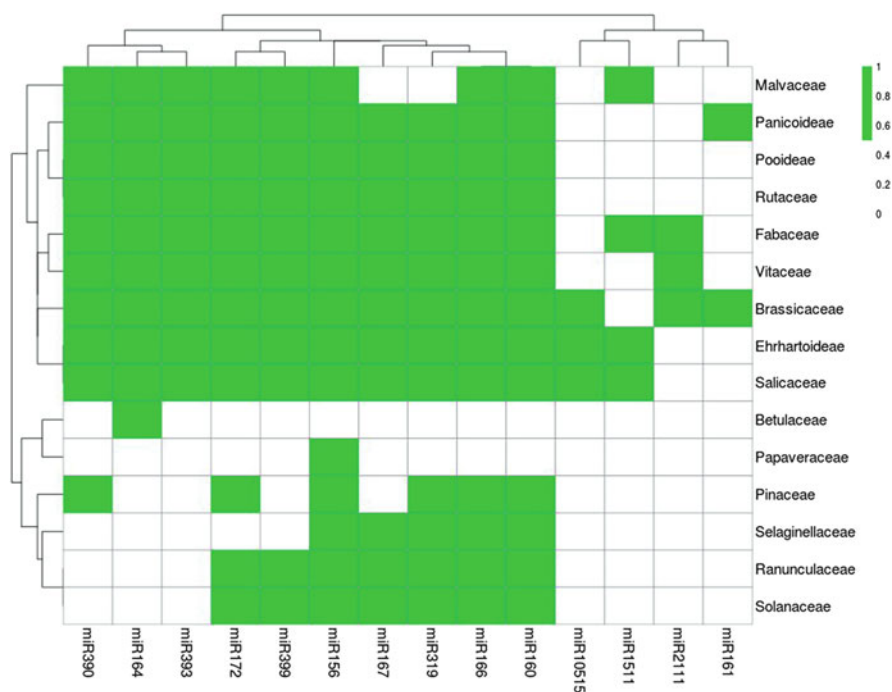


Fig. 3 microRNAs related to auxin metabolism found in the families studied. Families that have at least one microRNA, according to the plant microRNA database, were included. Clustering was performed according to the presence or absence of the microRNAs

4 Gene Regulation by MicroRNAs in Plants

MiRNAs were first discovered in *Caenorhabditis elegans* by Lee et al. (1993). Since then, miRNAs have become one of the most important and studied topics in biology (Cifuentes et al. 2010; Olmedo-Monfil et al. 2010; Cuperus et al. 2011; Djuranovic et al. 2012; Khraiweh et al. 2012; Manohar et al. 2013). Although much of the available information about the role of miRNAs has been done in *A. thaliana*, many miRNAs and their complementary sites in the targeted genes are conserved among angiosperms and gymnosperms (Table 1) (Floyd and Bowman 2004).

Although miRNAs are very small in size, they have big regulatory roles, e.g., regulating cell homeostasis during differentiation, organ development, cell death, and plant growth during normal or in vitro conditions (Bartel 2004, 2009; Jones-Rhoades and Bartel 2004; Mallory and Vaucheret 2004; Mallory and Vaucheret 2006). Bioinformatics tools have helped to discover that at least 30% of miRNA gene families, including miR156, 157, 158, 159, 163, 165, 166, 168, and 319, are present in approximately 10 different plant species and participate very actively in

different plant developmental processes (Jung and Park 2007; Reyes and Chua 2007; Sunkar and Zhu 2007; Li et al. 2012; Su et al. 2016).

Most of the miRNAs negatively regulate transcription factors, which have major roles in morphogenesis and development. For instance, miR156 targets the gene *SPL* (*Squamosa Promoter binding-Like*) involved in the regulation of the vegetative-to-reproductive transition as well as organ size (Wang et al. 2008; Wu and Poethig 2006). MiR164 targets the expression of *CUC1* (*CUP-SHAPED COTYLEDON*) and *NAC* (*NAM, ATAF1/2, and CUC2*) (Guo et al. 2005b; Raman et al. 2008) while miR165 and 166 regulate class III *HD-ZIP* genes (Bao et al. 2004; Carlsbecker et al. 2010; Furuta et al. 2012). *MYB* and *GAMYB*, which participate in the flowering process and seed germination, are targets of miR159 (Millar and Gubler 2005; Alonso-Peral et al. 2010). Another miRNA involved in flowering, specifically targeted *AP2* (*APETALA2*), is miR172 (Aukerman and Sakai 2003; Chen 2004). On the other hand, miR160 and miR167 regulate the expression of *ARF* (Wang et al. 2005), one of the most important transcription factor in regulating the expression of auxin response genes (Li et al. 2016). MiRNAs have become important signal molecules for auxin response, transport, and regulation.

5 MiRNAs in Auxins Signaling and Homeostasis

The precise mechanism of how auxins modulate plant growth and development is not fully understood. However, great progress has been made in the understanding of the signaling and transport of this PGR (McSteen 2010; Zhao 2010) (Table 3).

Strict control of auxin homeostasis and the maintenance of an appropriate level of IAA is important for normal growth and development. IAA is transported to the growing regions of a plant, and high IAA content correlates with intense cell division (Ljung et al. 2001; Tanaka et al. 2006). Proteins such as GRETCHEN HAGEN 3 (GH3), TRYPTOPHAN SYNTHASE β (TRP2), YUCCA (YUC), and others are important players in auxin homeostasis (Table 3).

In order to have a strict balance of IAA concentration in the cells, IAA is first distributed via phloem in a slow transport method from cell to cell, which is highly regulated by specific transport proteins such as AUXIN-RESISTANT 1/LIKE AUXIN-RESISTANT (AUX1/LAX), the ATP-BINDING CASSETTE SUBFAMILY B TRANSPORTER (ABCB), and the PIN-FORMED (PIN) (Petrášek et al. 2006; Cho et al. 2007; Petrášek and Friml 2009) (Table 3). PIN proteins are important components of auxin efflux, and its subcellular localization guides the flow of auxins (Tanaka et al. 2006). The polar movement of IAA allows a differential distribution, or gradients, of auxin within the plant tissues, and these gradients are dynamic during different developmental processes (Tanaka et al. 2006). The chemiosmotic transport of auxins is based on the differential pH between the apoplast (pH 5.5) and the cytoplasm (pH 7.0). IAAH is diffused throughout the plasmatic membrane or carried by the influx transport AUX1/LAX1 into the cell. Inside the cytoplasm, IAAH is dissociated to form IAA, which can be exported

Table 3 Proteins and genes required during the biosynthesis, transport and signaling of auxins

Proteins	Genes	References
<i>Biosynthesis</i>		
Tryptophan synthase β	<i>TRP2</i>	Bartel (1997)
Amidase	<i>AMI1</i>	Mano et al. (2010)
Tryptophan aminotransferase	<i>TAA1 TARI, 2</i>	Won et al. (2011)
Aldehyde oxidase	<i>AtAO1</i>	Ljung et al. (2002)
Flavin monooxygenase	<i>YUC1-11</i>	Zhao et al. (2001)
Cytochrome P450	<i>CYP79B2/3</i>	Zhao et al. (2002)
Nitrilase	<i>NIT1-4</i>	Cohen et al. (2003)
<i>Transport</i>		
Auxin influx transporter	<i>AUX1 LAX</i>	Kramer (2004), Zazimalová et al. (2010)
Auxin efflux carrier	<i>PIN1</i>	Gälweiler et al. (1998), Kramer (2004)
Serine threonine kinase	<i>PID</i>	Christensen et al. (2000)
ABC transporter	<i>ABCB1,19</i>	Zazimalová et al. (2010)
<i>Signaling</i>		
Aux/IAA transcription factor	<i>IAA1-25</i>	Hagen and Guilfoyle (2002)
Auxin response factor	<i>ARF1-23</i>	Guilfoyle and Hagen (2001, 2007)
F-box	<i>TIR1, AFB</i>	Dharmasiri et al. (2005), Parry et al. (2009)
Small Auxin Up RNA	<i>SAUR</i>	Hagen and Guilfoyle (2002)
<i>Conjugation</i>		
Gretchen Hagen 3	<i>GH3</i>	Hagen and Guilfoyle (2002)

from the cell by the efflux carriers PGP or PIN (Petrášek and Friml 2009; Robert and Friml 2009).

Auxins increase the early transcription of several genes such as *AUX/IAA*, *GH3*, and *SMALL AUXIN UP RNA (SAUR)* (Table 3). These genes can regulate plant physiology by modulating the interaction between transcription factors and the auxin response elements (AuxREs) of the involved genes (Abel and Theologis 1996) that normally are activated in the 2–20 min response (Guilfoyle et al. 1998). It is known that, under low nuclear concentration of auxin, the transcriptional repressors Aux/IAAs associates with the C-terminal domain of the ARF proteins, a class of transcriptional regulators that mediate the auxin-dependent response by binding directly to TGTCTC sequence of the auxin-responsive element (auxREs) found in the promoters of auxin-inducible genes (Kim et al. 1997; Reed 2001; Hagen and Guilfoyle 2002; Liscum and Reed 2002; Vernoux et al. 2011). When the nuclear concentration of auxin increases, AUX/IAA repressor interacts with TIR1/AUXIN SIGNALING F-BOX PROTEIN (AFB) required for recognition by CULLIN scaffold-type E3 ligases (SCF E3), and it is targeted for degradation (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Tan et al. 2007; Vernoux et al. 2011; Dinesh et al. 2016) (Table 3). The degradation of Aux/IAA induces the reactivation of the ARF function and the expression of the targeted genes involved in early auxin response.

Despite the fact that auxins have been studied biochemically, molecularly, and physiologically, it is still unknown how their regulation is coordinated in plant development. A small but important piece of evidence has proposed that the small RNAs are one of the responsible elements in the regulation of auxin homeostasis, transport, and signaling (Axtell 2013). There are many studies indicating that miRNAs have a major role in auxin genes related to homeostasis and signaling (Navarro et al. 2006; Gutierrez et al. 2009; Marin et al. 2010; Yoon et al. 2010; Si-Ammour et al. 2011; Chen et al. 2012; Kinoshita et al. 2012; Iglesias et al. 2014; Hrtayan et al. 2015), but there are still missing pieces in the picture of the role of miRNAs in plant development.

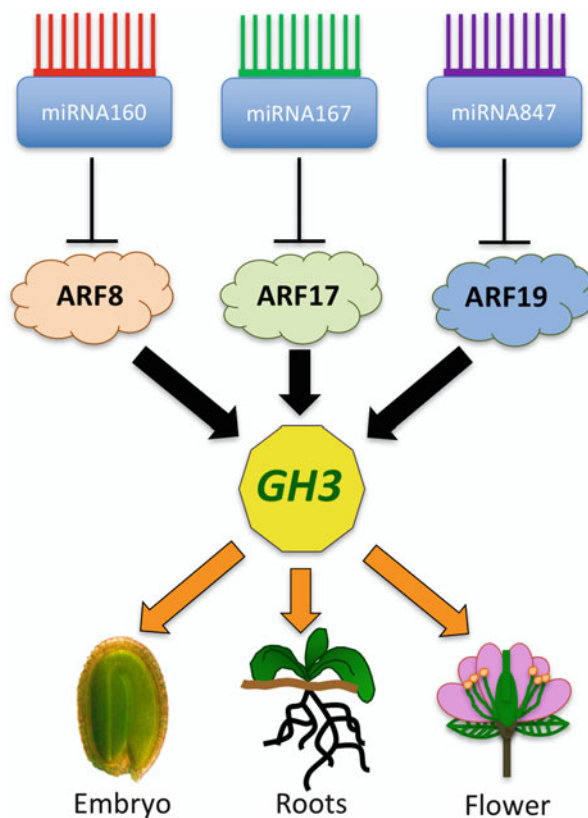
5.1 Auxin Homeostasis and MiRNAs

The homeostasis of auxins involves biosynthesis and degradation, as well as their conjugation with amino acids and carbohydrates. The hydrolysis of some of these conjugates liberates IAA (Ljung et al. 2002; Ljung 2013). The level of IAA concentration releases its signal transduction pathway and produces a molecular and biochemical response. This response begins with the sensing of IAA concentration through the assembly of a co-receptor complex that includes an F-box protein from the TIR1/AUXIN SIGNALING F-BOX PROTEIN (AFB) family and a member of the AUXIN/IAA-INDUCIBLE (AUX/IAA) family of transcriptional repressors. The homeostasis of IAA is mainly regulated by the GH3 proteins, which catalyze the conjugation of IAA with several amino acids to keep the auxin levels as normal as possible for each biological function (Bajguz and Piotrowska 2009; Chen et al. 2010).

It has been found that *GH3* genes are regulated by ARF8, ARF19, and ARF17 (Yang et al. 2006; Ding et al. 2013; Zhang et al. 2015). It is interesting to note that these ARF are targeted by miRNAs 167, 847, and 160, respectively (Table 2) (Mallory et al. 2005; Sorin et al. 2005; Yang et al. 2006; Ding et al. 2013; Wang and Guo 2015; Zhang et al. 2015; Li and Zhang 2016). Therefore, it seems that the final function of GH3 is a two-player regulation that depends not only on the ARF directly but also on the miRNAs that cleave specific ARFs' mRNAs (Fig. 4). For instance, ARF8 is involved in the regulation of the gene *GH3* during lateral root formation and hypocotyl elongation in Arabidopsis (Tian et al. 2004). ARF19 regulates the expression of *GH3* to mediate auxin homeostasis in lateral root formation (De Rybel et al. 2010). On the other hand, ARF17, which alters the expression of *GH3*, has been actively participating in embryonic, root, and floral development (Mallory et al. 2005) (Fig.4).

Although auxin transport is an important part of auxin regulation, the evidence for their involvement is very scarce. In a multicellular computational model, Muraro et al. (2014) found that miR165/166 can act as a regulatory mechanism for vascular patterning by targeting genes involved in auxin transport such as PIN1 (Muraro et al. 2014).

Fig. 4 microRNAs involved in ARF regulation for GH3 expression during embryo, root, and flower development. *ARF* AUXIN RESPONSE FACTORS, *GH3* GRETCHEN HAGEN 3



5.2 Auxin Signaling and MiRNAs

MiRNAs have also regulated the signaling pathway in response to auxin. While miRNA390 targets the expression of *TAS3* and this regulates ARF2, 3, and 4 - (Barrera-Figueroa et al. 2011), miR393 can target five different genes that belong to the TIR1 family (Sunkar and Zhu 2004), a positive regulator of auxin signaling. TIR1 targets the ARF transcriptional repressor, AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA), for degradation (Quint and Gray 2006; Tan et al. 2007).

Several miRNAs can downregulate some of the most studied classes of the ARF genes and direct their mRNA cleavage (Jones-Rhoades and Bartel 2004; Rhoades et al. 2002). For instance, miR167 targets *ARF6* and *ARF8*, while miR160 targets *ARF10*, *ARF16*, and *ARF17* (Rhoades et al. 2002; Kasschau et al. 2003; Allen et al. 2005; Mallory et al. 2005). Disrupting miRNA-mediated regulation of specific ARF can alter the normal phenotype. Regulation of *ARF17* mRNA levels by miR160 control is necessary for a proper transcriptional regulation of *GH3*-like early auxin response, which encodes IAA-amino acid-conjugating proteins (Mallory et al. 2005; Staswick et al. 2005; Park et al. 2007). Plants with five silent mutations

within the miR160-complementary domain of an ARF17 genomic clone, named 5mARF17, show an increase in the levels of expression of ARF17, reduced accumulation of GH3.5, and several floral defects (Mallory et al. 2005). Examples of these floral defects include accelerated flowering time, rosette serration, reduced petal size, abnormal stamen structure, and reduced fertility.

6 Role of MiRNA in Plant Growth and Development Mediated by Auxins

Recently, miRNAs appear to be key regulators that help to integrate diverse biological responses mediated by PGRs (Sanan-Mishra et al. 2013; Liu et al. 2016). Auxin signaling is highly regulated by miRNAs and appears to be conserved among different plant species, including *A. thaliana*, *Oryza sativa*, *Solanum lycopersicum*, and others (Rhoades et al. 2002; Eckardt, 2005; Hendelman et al. 2012; Sanan-Mishra et al. 2013). The first efforts led to the discovery that several ARF family members (*ARF8*, *ARF10*, *ARF16*, *ARF17*, and others) may be regulated by both miR160 and miR167 during early development in plants (Rhoades et al. 2002; Jones-Rhoades and Bartel 2004; Wang et al. 2005). Other crucial components of auxin signaling, such as *TIR1* and F-box auxin transcripts, have also been determined to be regulated by miR393 and miR394 (Allen et al. 2005; Jones-Rhoades and Bartel 2004). Interestingly, miR393 negatively regulates *TIR1*, *AFB2*, and *AFB3* transcripts to repress auxin signaling, thus increasing antibacterial resistance (Navarro et al. 2006) or helps to regulate auxin-related development of leaves by initiating the biogenesis of small interference RNAs to regulate the expression of *TIR* and *AFB2* (Si-Ammour et al. 2011). This suggests that miRNAs, and auxin levels, might regulate plant responses. Sorin et al. (2005) showed that Arabidopsis plants lacking ARGONAUTE1 (AGO1), a key player in the miRNA pathway, generate a super root, but are impaired in adventitious root formation. This impairment was connected to a defect in the regulation of auxin homeostasis. For instance, a study determined that a decrease in free IAA and its conjugates was correlated with downregulation of *GH3* genes that encode to acyl amidosynthetases (Hagen et al. 1991), which are also presumed to be targets of ARF17. Jain et al. (2006) found that high levels of *ARF17* mRNA are due to its resistance to cleavage by miR160; the same ARF that promotes the accumulation of *GH3* transcripts, including *GH3.2*, *GH3.3*, *GH3.5*, and *GH3.6*, are involved in the conjugation of IAA to amino acids (Jain et al. 2006). Therefore, a decrease of active IAA levels in the cell leads to dramatic pleiotropic defects, such as deformed embryos, abnormal stamens, and sterility, among others (Mallory et al. 2005). Many of these defects resemble phenotypes observed previously in Arabidopsis plants with mutations in *DCL1*, *AGO1*, *HYL*, and *HEN1* genes.

Five NAM/ATAF/CUC (NAC) domain transcription factor families are predicted to be under regulation by miR164. NAC1 acts downstream of *TIR1*,

transmitting auxin signals by promoting proper lateral root development. Guo et al. (2005a) showed that miR164 guides the cleavage of endogenous and transgenic *NAC1*. However, when *NAC1* is mutated to avoid the cleavage induced by miR164, *NAC1* messengers accumulate in the plant, allowing the production of more lateral roots (Guo et al. 2005b). Wang et al. (2005) determined that ARF10 and ARF16 are key controllers of root cap cell differentiation. They showed that miR160 overproduction in Pro35S:MIR160 plants and the ARF10-2 ARF16-2 double mutants display the same root tip defects such as uncontrolled cell division, impairment of cell differentiation, and loss of gravity sensing. This suggests that in the root auxin response, miR160 regulates the expression of ARF10 and ARF16 to promote the columnella proper cell differentiation (Wang et al. 2005). A similar effect was found in miR167, which regulates both female and male reproduction through the post-transcriptional control of ARF6 and ARF8. The overexpression of miR167 and the *ARF6ARF8* double mutants led to observation of similar and dramatic defects during the development of ovules and anthers. Therefore, miR167 is essential for regulating *ARF6* and *ARF8* during gynoecium and stamen development (Wu et al. 2006). Also, it has been found that transcripts of *ARF6* and *ARF8*, as well as *ARF17*, require additional levels of post-transcriptional regulation during the generation of adventitious roots. Gutierrez et al. (2009) showed that ARF6 positively regulates the amounts of both miR160 and miR167. ARF8 negatively regulates levels of miR167, whereas ARF17 regulates miR160 negatively, but positively affects the levels of miR167. Therefore, a complex regulatory mechanism apparently contributes to the regulation of adventitious root development, through a feedback regulation of miRNA homeostasis through direct and non-direct target TFs. A similar mechanism has been proposed between miR390 and *ARF4* during lateral rooting in *Arabidopsis* (Marin et al. 2010). In that model, miR390 expression senses external auxin concentration and directs the cleavage of the non-coding *TAS3* transcripts to affect the production of tasiRNA-*ARF* production. *ARF4* expression is critical for lateral root development, but interestingly inhibits miR390 expression through a negative regulation between the tasiRNA-*ARF* pathway and ARF4, which allows the spatiotemporal expression of *ARF4* (Yoon et al. 2010). In another study, it was shown that there is a positive and negative feedback regulation of miR390 by ARF2, ARF3, and ARF4, which regulate the miR390 expression pattern. This regulatory network allows the maintenance of optimal levels of the transcripts of *ARF* and thus specifies the timing of lateral root growth (Marin et al. 2010).

On the other hand, the regulation of shoot regeneration in vitro also requires the participation of miRNAs. By using *Arabidopsis* calli, it was found that non-totipotent cells contain more miR160 transcripts than a totipotent line. Taken into account that *ARF10* is a target of miR160, Qiao et al. (2012) showed that transgenic plants with an miR160-resistant form of *ARF10* increased shoot regeneration up to fivefold. In contrast, shoot regeneration was practically null when miR160 was overexpressed. More recently, deep small RNA sequencing during embryo differentiation in maize allowed the detection of more than 100 known miRNAs belonging to 23 miRNA families (Shen et al. 2013), where several of them

could be involved in the establishment of embryogenic calli. It appears that the initial callus formation requires a major transcriptional regulation due to the high levels of differentially expressed miRNAs found in the callus (Shen et al. 2013). Moreover, the participation of miR393, which targets *TIR1*, and both miR160 and miR167, which target ARF messengers, seem to be important during the early stages of somatic embryogenesis. For instance, the overexpression of miR167 inhibits somatic embryo generation by altering the auxin response and auxin transport in the embryogenic calli (Su et al. 2016). This study also found that *ARF6* and *ARF8* transcripts regulated by miR167 are necessary for SE since both TFs are required in auxin signaling pathways and can mediate auxin-induced gene activation during the SE induction.

Taken together, these results clearly show that miRNAs play a crucial role during plant growth and that their participation in the auxin signaling pathway is necessary to help to coordinate plant development by controlling the auxin response genes properly.

7 Concluding Remarks

Since auxins are involved in almost every aspect of the physiology of plants, the balance of auxin concentration is crucial in producing such a diversity of responses. Most of these responses are regulated by miRNAs. The miRNA databases grow daily, and more and more of their roles are discovered. However, a deeper understanding of their function is needed for a complete model of action of the miRNAs. The highly networked regulation that miRNAs exhibit during root development, embryo formation, and in auxin signaling generally throughout the plant increases the complexity of our understanding of PGR. There are a couple of miRNAs, such as 160 and 167, that can regulate many ARFs. However, a more detailed knowledge of the miRNAs involved in specific target degradation in particular processes would help to understand the communication and balance among all players. For instance, it is necessary to understand the role of miRNAs in the epigenetic regulation of differentiation and somatic embryogenesis in order to outline a signaling map to increase the efficiency of the process in some recalcitrant species.

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