Chapter 12
The Role of Small RNAs in Plant Somatic Embryogenesis

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Abstract In plants, differentiated somatic cells can revert their identity to pluripotent, reprogrammed cells in order to optimize growth and development depending on external conditions and in aid of overcoming their limitations as sessile organisms. Different modes of regeneration include tissue repair, de novo organogenesis and somatic embryogenesis (SE). The latter usually comprise the formation of proliferating pluripotent cell masses called callus. Identification and characterization of genes involved in the SE process allows the exploitation of distinctive features that make a tissue susceptible to change its normal cell fate and produce new plants massively.

Small RNAs (sRNAs) are non-coding RNA (ncRNA), 20–24 nucleotides long molecules involved in plant development, reproduction and genome reprogramming. Likely, the enormous variety of operating sRNA pathways contributes to the plant phenotypic plasticity. Two main sRNAs classes are defined by their modes of biogenesis: a class in which the precursor is a single-stranded, hairpin loop forming RNA (hpRNA), mainly represented by microRNAs (miRNAs) and a class in which the precursor is a dsRNA molecule (dsRNA) comprising several small interfering RNAs (siRNAs).

sRNAs, especially miRNAs, are common regulators of transcription factors (TFs) essential for plant meristem maintenance, growth and proliferation control, and with recently uncovered role in somatic to embryonic cell reprogramming. Although the siRNA function in plant development and SE has been much less explored, recent findings shape out their relevance in organ patterning and stress responses, both involved in cell plasticity. This review focuses on compiling and integrating the described function of miRNAs and siRNAs as a molecular basis in establishing cell dedifferentiation and further plant regeneration in economically relevant crops.

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12.1 The SE Process

12.1.1 General Description

Somatic embryogenesis (SE) is an alternative plant reproduction process where embryos are produced from somatic tissues through an initial cell dedifferentiation promoted by exogenous signals (Elhiti et al. 2013). Dedifferentiated cells are able to proliferate maintaining their totipotential state (embryogenic masses or callus) and can further develop into a whole plant if the exogenous signal is removed.

SE has great impact on plant biotechnology and is widely used for clonal propagation, transformation or somaclonal variation. In addition it constitutes a valuable model to study early developmental features of embryogenesis, molecular aspects of cell transition during differentiation and hormone responses (De-la-Peña et al. 2015).

While zygotic embryogenesis initiates upon fertilization and comprises a series of molecular events underlying morphogenesis and embryo patterning, SE results from differentiated somatic tissues, which gain on embryogenic competence as a response to imposed external stimuli. For example, in maize, immature embryos have proven to display greater competence to achieve totipotency. In this plant species embryogenesis commitment requires dedifferentiation and further establishes cell proliferation prior to plant regeneration (Garrocho-Villegas et al. 2012).

12.1.2 Known SE Markers

The first step in SE induction is cell dedifferentiation where the cell fate of particular differentiated cells returns to a totipotent ground state as a response to external stimuli. The process is related to stressful conditions, such as temperature change, high phytoregulator concentration, light deprivation and others (Elhiti et al. 2010; Kumar and Van Staden 2017). The imposed stress and exogenous hormones induce gene expression reprogramming, particularly through AUXIN RESPONSE FACTORS (ARFs), AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA), Helix-Loop-Helix (bHLH), LATERAL BOUNDARY DOMAIN (LBD) and other transcription factors (TFs) to direct cells towards dedifferentiation (Yang et al. 2012; Elhiti et al. 2013; Ge et al. 2016).

Upon promoting dedifferentiation, the achievement of embryogenic potential is crucial for further plant regeneration through SE. Several markers correlate with enhanced embryogenic potential. These include LEAFY COTYLEDON (LEC1 and LEC2), WUSCHEL (WUS) and BABY BOOM (BBM) genes (Su et al. 2009; Elhiti et al. 2010; Lowe et al. 2016). LEC1 and LEC2 are required to activate endogenous auxin biosynthesis, which consequently up-regulates the expression of WUS and SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) (Elhiti et al. 2013).

Induced totipotent embryogenic tissues further activate signalling towards cell division and meristematic fate. Genes involved in successful proliferation program include cell cycle regulators and signal transduction components. Exogenous phytohormones, present in the callus proliferation medium, contribute to enhanced SHOOT APICAL MERISTEM (STM), CYCLIN DEPENDENT KINASEs (CDKs) and WUS gene expression (Elhiti et al. 2010). On the other hand, negative regulators of WUS, such as CLAVATA (CLV1, CLV2 and CLV3) repress meristematic cell proliferation and promote differentiation (Elhiti et al. 2013).

More recently, small RNAs (sRNAs) have emerged as master regulators for most of the above-mentioned transcription factors. Their role in the SE process has been intensively studied in model and agronomical plant species over the last decade (Luo et al. 2006; Shen et al. 2013; Wu et al. 2015; Szyrajew et al. 2017).

12.2 sRNAs Classification, Biogenesis Pathways and Functions

12.2.1 sRNA Classification

Major sRNA groups include small interfering RNAs (siRNAs) and microRNAs (miRNAs). Further classification separates siRNAs in: hairpin-derived siRNAs (hp-siRNAs), natural antisense siRNAs (nat-siRNAs), secondary siRNAs and heterochromatic siRNAs (hc-siRNAs). miRNAs and hp-siRNAs derive from single-stranded RNA precursors that form a stable hairpin loop, while other siRNAs originate from double-stranded RNA (Axtell 2013). Plant genomes usually present several individual genes encoding miRNAs from the same family and in few cases the miRNA originates from transcripts of protein-coding genes (reviewed in Budak and Akpinar 2015). Secondary siRNAs include phased- and trans-acting siRNAs. Phasing is a consequence of successive DCL processing that initiates at particular site within the dsRNA precursor determined by specific miRNA targeting (Fig. 12.1). Secondary siRNAs that act *in trans* to direct silencing of distinct mRNA targets are termed tasiRNAs. Most known tasiRNAs are also phased (Axtell 2013). Heterochromatic siRNAs commonly derive from plant transposable elements (TEs) and trigger important epigenetic mechanisms (Borges and Martienssen 2015).

12.2.2 sRNA Biogenesis Pathways

All sRNA production requires DICER-LIKE (DCL) enzymes to produce 21–24 nt long RNA duplexes with 2 nt overhangs at the 3′ ends from precursors. Duplexes are protected by 2′-methylation at their 3′ ends by HUA ENHANCER 1 (HEN1). These duplexes are recognized by ARGONAUTE (AGO) proteins in complex with

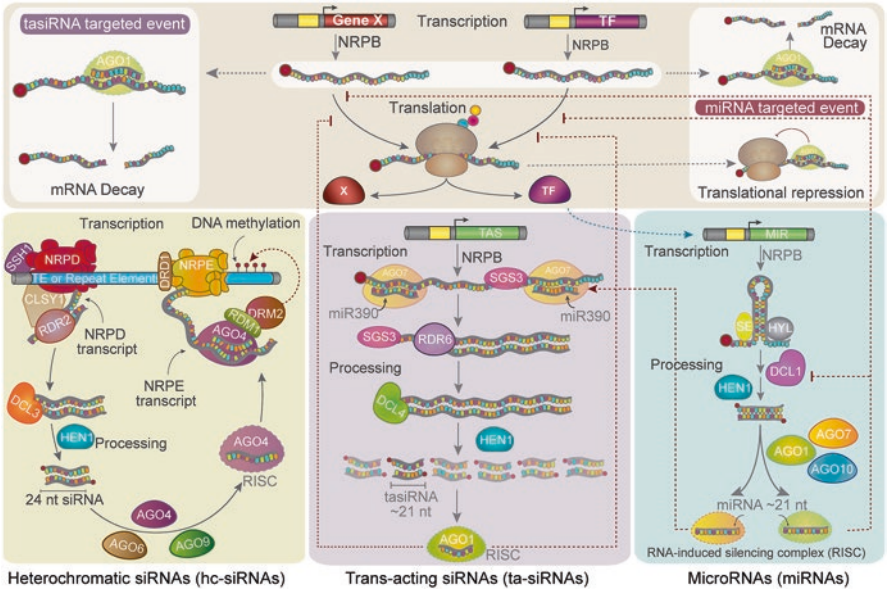

Fig. 12.1 Small RNA biogenesis pathways, interconnection and mechanisms of action. Lower panels represent the distinct origin (genetic loci) and biogenesis of hc-siRNAs, tasiRNAs and miRNAs (from left to right). The upper panel represents distinct modes of action and targets for tasiRNAs and miRNAs. Interconnection between miRNAs, tasiRNAs and targeted transcription factors (TFs) is shown by dotted lines. NRPB, RNA Pol II; NRPD, RNA Pol IV; NRPE, RNA Pol V; DCL, Dicer-like; AGO, Argonaute; RDR, RNA-dependent RNA polymerase; HEN1, sRNA methylase HUA enhancer 1; DRM2, domain rearranged methylase 2

other partners to select the mature sRNA strand and target protein-coding or non-coding RNAs by sequence complementarity. There are several comprehensive reviews on plant sRNA production and action (Bologna and Voinnet 2014; Borges and Martienssen 2015). Commonly, sRNA-charged AGO constitutes an RNA-induced silencing complex (RISC) that usually exerts post-transcriptional gene silencing (PTGS) by either transcript degradation or translational inhibition (Rogers and Chen 2013). However, the particular subclass hc-siRNAs promotes transcriptional gene silencing (TGS) and RNA-directed DNA methylation (RdDM), which is very important for TE control in plants. sRNAs originating from dsRNA precursors require the action of RNA-dependent RNA polymerase (RDR) and other stabilizing proteins for their biogenesis. The pathways depicted in Fig. 12.1 show major steps and particular DCL, AGO and RDR family members, as well as other enzymes required for miRNA, tasiRNA and hc-siRNA production, genetically dissected in the model plant *Arabidopsis thaliana*. Several mutants for these proteins have also been identified in agronomical crops such as rice and maize, unravelling specialized functions for some of them (Nagasaki et al. 2007; Nobuta et al. 2008; Chitwood et al. 2009; Thompson et al. 2014).

12.2.3 sRNA Mechanisms of Function

Plant microRNAs regulate target RNAs by nearly perfect complementarity with sequences within any region of the transcript (Axtell 2013). miRNA-guided RISC preferentially induces target cleavage generating fragments at the targeted sequence that could follow up experimentally by degradome analyses (Ding et al. 2012; Yang et al. 2013). However, there are several examples of targets reduced at protein level, but not affected at mRNA level, due to miRNA action (Chen 2004; Brodersen et al. 2008; Beauclair et al. 2010). These studies suggested translational repression as a second way of action for plant miRNAs (Fig. 12.1). The extent of miRNA-target complementarity has been considered as premise to turn the balance towards either slicing (perfect) or translational repression (imperfect) in animals. However, the two modes of miRNA action have been shown to simultaneously operate in plants independently of the grade of complementarity (Aukerman and Sakai 2003; Beauclair et al. 2010).

Trans-acting small interfering RNAs (tasiRNAs) derive from precursor TAS genes transcribed by RNA pol II (Fig. 12.1). A miRNA drives the initial processing; the cleaved fragment is converted to dsRNA by RDR6 and sliced by DCL4 to 21-nucleotide siRNAs in a phased arrangement from the miRNA cleavage site (Xia et al. 2017). Four different TAS genes have been identified in *Arabidopsis thaliana* (Fei et al. 2013), but TAS3 is the most conserved and well-studied in different plants. Initial cleavage of TAS3 transcript is promoted by miR390 charged on AGO7, and some of the derived tasiRNAs target several members of the ARF3/4 family. This pathway is known as miR390-TAS3-ARF and related tasiRNAs are termed tasiR-ARFs (Dotto et al. 2014).

Heterochromatic siRNAs (hc-siRNAs) originate from repeat-rich loci and TEs. Initial transcription by plant-specific RNA Pol IV (NRPD) and conversion of the transcript to dsRNA by RDR2 are required (Matzke and Mosher 2014). Then DCL3 processes the precursor to 23–24 nt duplexes, which are exported to the cytoplasm where they are loaded onto members of the AGO4 clade and returned to the nucleus (Borges and Martienssen 2015). AGO4-hc-siRNAs are recruited to homologous loci transcribed by plant RNA Pol V (NRPE) to deposit repressive chromatin marks, such as 5-methyl cytosine at asymmetric CHH context and histone H3K9 methylation. Usually 23–24 nt long hc-siRNAs represent the most abundant sRNA class in many plant species. In maize, mutation of RDR2 causes important reduction of 23–24 nt hc-siRNAs accompanied by increase of 21–22 nt siRNAs, including some miRNAs and tasiRNAs (Nobuta et al. 2008). This and other loss-of-function mutants in the RdDM pathway are not associated with major developmental defects suggesting that transcriptional silencing involves several layers of regulation.

12.3 miRNA Role in Plant Somatic Embryogenesis Induction

microRNAs are important regulators of plant developmental switches. Their role in SE induction relies on targeting central TFs that determine tissue differentiation. They also act as sensors of imposed stress conditions during dedifferentiation, phytohormone signalling and responses, as well as in embryogenic potential acquisition. In *Arabidopsis thaliana*, miR165 and miR166 target the CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III) TFs PHABULOSA (PHB) and PHAVOLUTA (PHV), which are positive regulators for LEC2 expression. On the other hand, miR160 regulates ARF10, ARF16 and ARF17 involved in auxin signalling during SE induction (Wójcik et al. 2017). In addition, miR393 controls the levels of auxin receptors TIR1 and AFB2 (Wójcik and Gaj 2016). The relevance of these miRNAs in the context of auxin signalling pathways is described with more details below.

A pioneer study developed in rice revealed that some miRNAs are particularly enriched in dedifferentiated tissues (Luo et al. 2006). Such finding was followed by reports based on next-generation sequencing (NGS) techniques approaching miRNA abundances during SE embryogenesis induction, plant regeneration or between tissues with distinct embryogenic potential (Shen et al. 2013; Wu et al. 2015; Szyrajew et al. 2017). A common finding for these studies was that development-related miRNAs (miR156, miR159, miR164, miR166 and miR172) tend to decrease their levels upon dedifferentiation, while stress-related (miR319, miR396, miR397, miR398 and miR408) increase (Fig. 12.2). Other miRNAs related to auxin responses (miR160, miR167, miR169 and miR390) may show transient increases depending on the stage of dedifferentiation induction. On the other hand, during plant regeneration through SE, stage-specific miRNA patterns and their target regulation oppose the dedifferentiation status revealing important roles for miR156, miR159, miR164 and miR168 in *Citrus sinensis* (Wu et al. 2011), *Larix leptolepis* (Zhang et al. 2012), *Dimocarpus longan* (Lin and Lai 2013) and *Zea mays* (Chávez-Hernández et al. 2015). However, it is important to highlight that each plant species requires particular in vitro culture conditions and some of the conserved miRNAs might display species-specific patterns.

12.4 Relevance of sRNAs in Auxin Responses and Homeostasis

12.4.1 The Auxin Signal Transduction Pathway

Auxins are the most widely studied phytoregulators in plants (Sanan-Mishra et al. 2013). They are involved in plant growth, cell division, elongation and differentiation, apical-basal axis formation, embryogenesis, meristem formation and tropism (Hrtyan et al. 2015; Kasahara 2016; Mutte et al. 2018). Indole-3-acetic acid (IAA)

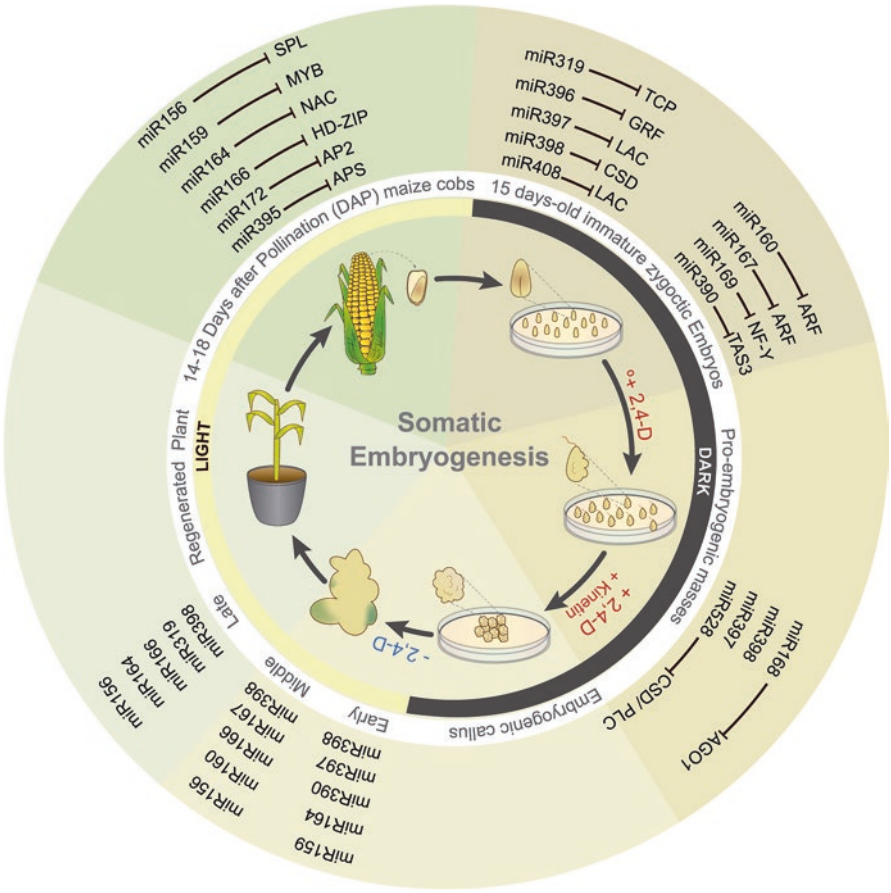

Fig. 12.2 sRNAs role in somatic embryogenesis (SE) and plant regeneration (exemplified with maize). SE induction and dedifferentiation is represented at the top of the circle using as explant immature embryos and 2,4-D/darkness as stimulus. Further embryogenic callus proliferation establishment includes kinetin in addition to 2,4-D (Garrocho-Villegas et al. 2012). The bottom part represents differentiation induction of proliferating callus by phytoregulators removal in the presence of photoperiod. The circle is completed by plant regeneration and reproduction. At each stage, the most abundant miRNAs detected for maize and other plant species (details in Table 12.2) are shown on the external circle together with their proposed roles in regulating TFs or proteins crucial for the SE process

is the most common natural auxin and the final product of general auxin biosynthesis mechanisms. There are also synthetic auxins: 2,4-dichlorophenoxy acetic acid (2,4-D) and 1-naphtaleneacetic acid (NAA). 2,4-D mimics IAA in the perception and signalling, except for cell-to-cell auxin transport mechanisms (Fukui and Hayashi 2018; Mutte et al. 2018).

Auxin regulatory networks operate by three dynamic processes: (1) auxin biosynthesis and inactivation; (2) cell-to-cell auxin transport (auxin polar transport)

and (3) final signal transduction (Fukui and Hayashi 2018). The first two are related with the balance of auxin concentrations in specific tissues at certain developmental stages, whereas the third one represents the final response to auxin perception, which consists in the transcriptional activation or repression of a wide range of genes (Sanan-Mishra et al. 2013).

Exogenous auxin influx takes place by passive diffusion or by AUXIN RESISTANT 1/LIKE AUXIN (AUX1/LAX) transporters (Fig. 12.3). Auxin efflux is carried out through PIN-FORMED (PIN) efflux carriers and ATP-BINDING CASSETTE subfamily B/MULTIDRUG RESISTANCE/P-GLYCOPROTEIN (ABCB/MDR/PGP). The influx/efflux conforms the auxin polar transport system responsible to maintain auxin levels and gradients between cells. The transporters (AUX/LAX, PIN and ABCB) have particular spatiotemporal expression and subcellular localization to determine the specific auxin gradients during plant growth and development (Barbosa et al. 2018; Fukui and Hayashi 2018; Zhao 2018).

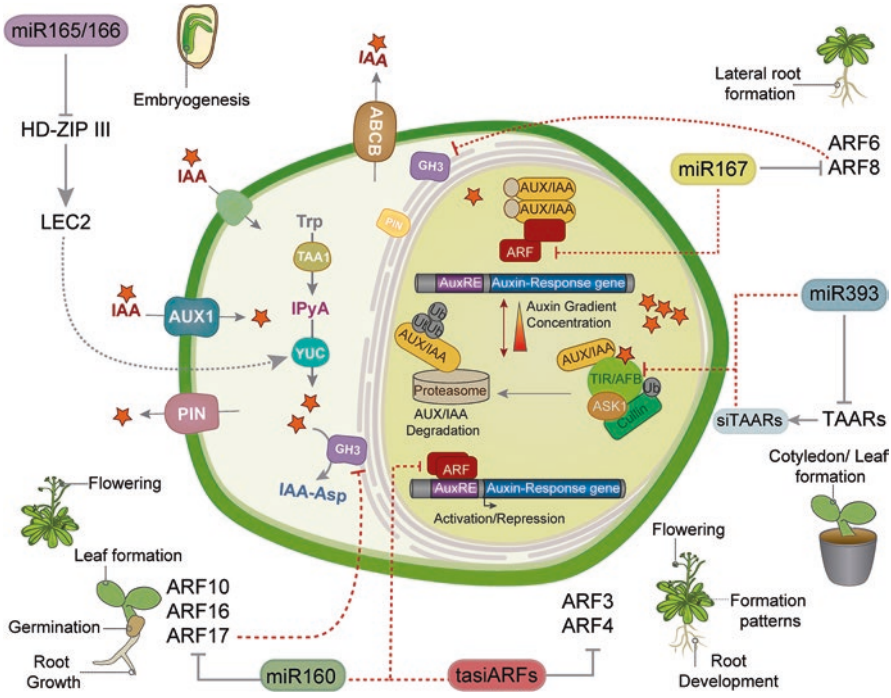

Fig. 12.3 sRNA-mediated regulation on plant auxin signalling pathway**.** The image represents the main Arabidopsis auxin signal transduction pathway explained in Sect. 12.4. miR160 controls IAA degradation by inhibiting ARF10/16/17 during seed germination and plantlet establishment. miR165/166 indirectly affects IAA biosynthesis by repressing HD-ZIP III TFs which are required to promote LEC2 and YUC expression. miR167 inhibits ARF6/8 involved in auxin-responsive gene expression, particularly during lateral root formation. miR393 represses TAAR expression and promotes generation of TAAR-derived siRNAs during cotyledon leaf formation. Opposite gradients of tasiR-ARFs and their ARF3/4 targets help to establish leaf pattern formation, appropriate root development and flowering

Auxin biosynthesis and inactivation has been characterized mostly in *Arabidopsis thaliana*. However, homologous pathways are highly conserved in plants. A Trp-dependent pathway produces endogenous IAA by Trp conversion in two sequential steps: (1) Trp is converted to indole 3-pyruvic acid (IPyA) by the TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS1/TRYPTOPHAN AMINO TRANSFERASE RELATED (TAA1/TAR gene family); (2) Enzymes from the flavin monooxygenase family YUCCA (YUC) catalyse the conversion of IPyA to IAA (Fig. 12.3). The second conversion is the rate-limiting step because of strict YUC availability and spatiotemporal regulation. Auxin catabolism and inactivation are as well important to maintain optimal endogenous levels for certain processes. The Gretchen Hagen 3 (GH3) family of IAA-amide synthetases conjugate IAA to amino acids and in such form the auxin can be degraded. Conjugation is reversible depending on the amino acid identity (Kasahara 2016; Fukui and Hayashi 2018; Zhao 2018).

Auxin signal transduction triggers transcriptional regulation of many gene families. It involves intracellular receptors, transcriptional activators and repressors (ARFs) and auxin-responsive genes. The TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALLING F-BOX (TIR1/AFB) receptor family are responsible for the perception of intracellular auxin levels. TIR1 is an F-box protein that conforms the SCF-type complex (Skp1, Cullin, and F-box protein-type), which act as ubiquitin-ligases responsible for protein ubiquitination and further degradation via the proteasome (Sanan-Mishra et al. 2013; Wang et al. 2018). At high auxin levels, TIR1/AFB receptors promote interaction between the SCF-type complex and the ARF inhibitors AUX/IAA. Such interaction promotes AUX/IAA degradation and ARF release to regulate auxin-responsive genes bearing DNA cis-elements (AuxRE) in their promoters (Leyser 2018; Kim et al. 2018; Wang et al. 2018). Auxin-responsive genes include GH3s, SAURs (SMALL AUXIN UP-REGULATED RANKs) and AUX/IAA. Gene repression or activation depends on ARF identity (Sanan-Mishra et al. 2013; Roosjen et al. 2018).

12.4.2 miRNAs Related to Auxin Responses

From the 23 ARF family members identified in *Arabidopsis thaliana*, at least five are regulated by miRNAs (Mallory et al. 2005). Arabidopsis miR160 targets ARF10, ARF16 and ARF17. The loss of miR160 target site in any of these ARFs results in developmental defects, such as reduced ABA sensitivity during germination (ARF10), defective root cap development and alteration in lateral root formation (ARF10 and ARF16); embryo symmetry anomalies, leaf shape defects, premature and abnormal inflorescence development and root growth impairment (ARF17) (Mallory et al. 2005; Wang 2005; Liu et al. 2007). Interestingly, ARF17 represses GH3 transcription, thereby affecting the intracellular auxin inactivation (Fig. 12.3). In addition, miR167 represses ARF6/ARF8, which also regulate GH3 transcription. Surprisingly, it has been reported that miR167 promoter displays AuxRE elements probably regulated by other ARFs. Therefore, miR160/167 nodes seem to have

complementary roles at least in root development (Rubio-Somoza and Weigel 2011; Sanan-Mishra et al. 2013; Hrtyan et al. 2015).

Another miRNA involved in auxin regulation is miR393. The targets of this miRNA belong to the family of F-box proteins, including four members of the TIR1/AFB2 clade of auxin receptors (TAARs). Cleavage of TAAR transcript promotes the production of secondary siRNAs (siTAARs) which regulate the final expression of each TAARs from where they were originated and other unrelated genes, generating auxin accumulation and developmental abnormalities of leaves and cotyledons (Si-Ammour et al. 2011; Singh et al. 2018). On the other hand, miR165/166 indirectly affect IAA biosynthesis through inhibiting the activation of YUC transcription by LEC2 (Fig. 12.3; Wójcikowska et al. 2013; Wójcik et al. 2017).

12.4.3 tasiRNAs Involved in Auxin Responses

As described in Sect. 12.2, tasiRNAs targeting ARF3/4 are commonly known as tasiR-ARFs (Dotto et al. 2014; Xia et al. 2017). The tasiR-ARF highly conserved regulatory mechanism is required for proper leaf development, as well as juvenile to adult phase changes (Guilfoyle and Hagen 2007). Other functions are related to flower development and lateral root formation under normal and salt stress conditions (Marin et al. 2010; Hrtyan et al. 2015; He et al. 2018). Before lateral root initiation, miR390 expression is activated in xylem cells and promotes tasiR-ARFs production to repress ARF3/4 transcripts in the new primordium. This provokes endogenous auxin level alteration at particular sites, required for lateral root formation and appropriate plant growth (Marin et al. 2010). A recent report in Poplar (Populus spp.) showed that osmotic stress inhibits auxin signalling to enhance lateral root formation through miR390 expression stimulation and tasiR-ARFs accumulation (He et al. 2018).

12.5 Other siRNAs in Cell Dedifferentiation and Proliferation Establishment

12.5.1 Epigenetic Regulation in SE

Epigenetic mechanisms coordinate gene reprogramming for the acquisition of totipotency during dedifferentiation of somatic cells (Miguel and Marum 2011; Elhiti et al. 2013). Such reprogramming is partly achieved by DNA methylation and histone modifications in response to environmental and stress conditions to achieve the developmental switching in somatic cells as adaptation to the external cues (Henderson and Jacobsen 2007; Huettel et al. 2007; Neelakandan and Wang 2012).

DNA methylation is an essential epigenetic mechanism that regulates and maintains gene expression programs (Milutinovic et al. 2003). In plants, cytosine methylation occurs in the context of CG, CHG and CHH (H = A, T or C) and is catalysed by METHYLTRANSFERASE (MET), CHROMOMETHYLASE (CMT) and DOMAIN REARRANGED METHYLTRANSFERASE (DRM) (Cao and Jacobsen 2002; Fehér 2015). Several factors influence DNA methylation during plant SE (Elmeer and Hennerty 2008; Joshi et al. 2008) and both, hyper- and hypomethylation, play crucial roles in somatic embryo development (Chakrabarty et al. 2003; Nic-Can et al. 2013). Early research in plant SE determined that high auxin concentrations and nitrogenous compounds like L-proline, commonly present in in vitro culture media, affect the DNA methylation status, and hence gene expression (LoSchiavo et al. 1989). DNA methylation inhibition correlates with SE competence impairment and loss of regeneration capacity in *Medicago truncatula* (Santos and Fevereiro 2002) and *Daucus carota* (Yamamoto et al. 2005). Therefore, certain levels of DNA methylation have to be maintained for a proper course of SE (De-la-Peña et al. 2015).

Chromatin remodeling has been widely reported for plant somatic cell dedifferentiation, organogenesis, embryogenesis and regeneration (Grafi et al. 2007; Valledor et al. 2010). This process allows TFs and chromatin modifiers to access DNA and exert gene expression control. The regulation is mediated by particular histone N-terminal methylations, acetylations, ubiquitinations and phosphorylations (Kouzarides 2007). During SE induction in *Coffea canephora* the H3K9me2 repressive mark was absent, while H3K4me2 and H3k4me3 activation marks increased. Additionally, after the first week of induction, the levels of H3K27me2 and H3k27me3 repressive marks were also substantially reduced (Nic-Can et al. 2013). Reduction in repressive histone modifications has been associated with genes encoding TFs involved in cell differentiation, such as BBM1, LEC1 and WUSCHEL-RELATED HOMEOBOX4 (WOX4) to promote successful SE induction (Lafos et al. 2011). In addition, a mutant for the chromatin modifier PRC2, which directly binds H3K27me3 and promotes repressive chromatin remodeling, tends to develop embryo-like structures from differentiated tissues (Ikeuchi et al. 2015). This supports the role of transcriptional repression in preventing dedifferentiation of mature somatic cells and suggests de-repression is needed to achieve cellular dedifferentiation and SE progression. Although all these studies have pointed out the relevance of dynamical plant chromatin regulation during SE, the mechanisms underlying the epigenetic plasticity required for cell totipotent status have been still poorly explored.

12.5.2 sRNA Impact on Epigenetic Landscapes

Plant hc-siRNAs (Fig. 12.1) are involved in heterochromatin formation and transcriptional gene silencing by guiding sequence-specific DNA and histone methylation through RdDM (Matzke and Mosher 2014; Borges and Martienssen 2015). In

Arabidopsis thaliana, RdDM targets genomic loci for de novo DNA methylation through DRM2 (Zhang and Zhu 2011; Saze et al. 2012). Reports from several plant species have illustrated hc-siRNA-mediated epigenetic regulation, their role in chromatin organization and transcription silencing during different developmental stages and stimuli. It has been shown that hc-siRNAs participate as mobile elements for inter-tissue epigenetic regulation. In grafting experiments using wild-type and mutant plants, unable to produce hc-siRNAs, the movement of these sRNAs was detected from wild-type-to-mutant tissues to induce *novo* DNA methylation (Molnar et al. 2010; Tamiru et al. 2018). Also, hc-siRNAs mediate transgenerational epigenetic regulation. Prior fertilization, cells surrounding germline undergo DNA demethylation leading to the transcriptional activation of endogenous TEs (Zemach and Zilberman 2010). TE reactivation triggers the formation of hc-siRNAs that move into the germ cells and ensure epigenetic silencing of TEs in the embryo (Olmedo-Monfil et al. 2010; Kumar and Van Staden 2017). Also, mutants for components of hc-siRNA biogenesis were related to decondensation of pericentromeric repeats and depletion of H3K9me2 at chromocenters leading to genome instability (Pontes et al. 2009). The relationship between RdDM and chromatin remodelers has been demonstrated in maize (Fu et al. 2018). Mutants for CMT or the nucleosome remodeler DDM1 exhibited decrease in RdDM activity and nearly complete loss of both, 24 nt hc-siRNAs and CHH-methylation. Curiously, the loss of 24 nt hc-siRNAs was accompanied by a dramatic increase of 21 and 22 nt siRNAs mapping to heterochromatic loci in the genome. However, these siRNAs apparently are unrelated to DNA methylation and RdDM.

12.5.3 hc-siRNAs and Other siRNAs during SE

Despite all reports that have linked epigenetic regulation by sRNAs with plant development, to date very few studies have approached the implication of hc-siRNAs and other siRNAs in SE regulation. While investigating sRNA roles in synchronic SE of *Larix leptolepis*, an overrepresentation of 24 nt siRNAs was observed for synchronous embryos suggesting their participation in SE synchronism, a crucial hallmark in plant tissue culture (Zhang et al. 2014). Likewise, genome-wide analysis of sRNAs in non-embryogenic and embryogenic tissues of 'Valencia' sweet orange (*Citrus sinensis*) SE indicated that 24 nt siRNAs exhibited lower abundance in the non-embryogenic callus (Wu et al. 2015). In addition, plant regeneration through rice SE revealed DNA hypomethylation associated with 24 nt hc-siRNAs loss (Stroud et al. 2013). Also, in immortalized Arabidopsis cell suspension cultures, particular heterochromatic regions were hypomethylated and TEs became activated (Tanurdzic et al. 2008). However, the 24 nt hc-siRNAs were significantly reduced only for particular TEs.

In maize, the 24 nt sRNA population importantly decreased during the establishment and maintenance of embryogenic callus for the Tuxpeño VS-535 cultivar (Alejandri-Ramírez et al. 2018). However, 21–22 nt populations were not affected. Interestingly, the 24 nt-long hc-siRNAs derived from retrotransposons decreased

only transiently during callus proliferation establishment, concomitant with 22 nt increases. Unexpectedly, such changes were accompanied by reduction in the expression of some transposons, suggesting that TE regulation might be needed for proper establishment of embryogenic callus and the acquirement of proliferative status. Moreover, the role of other maize siRNAs was revealed while studying factors determining the frequency of embryonic callus formation in the Chinese maize inbred line 18-599R (Ge et al. 2017). Surprisingly, some 24 nt siRNAs mapping to promoter gene regions were significantly up-regulated and correlated with hypermethylation of the corresponding target genes during different stages of embryogenic callus induction and formation. This further resulted in decreased expression of the target genes. All these data only expose the tip of an iceberg that represents the largely unknown role of hc-siRNAs and other siRNAs for gene expression regulation during plant SE. Whether these siRNAs act through the RdDM pathway remains to be demonstrated.

12.6 sRNAs in Plant Regeneration Through Somatic Embryogenesis

12.6.1 Comparison Between Somatic and Zygotic Embryogenesis

Numerous studies have shown the resemblance between somatic and zygotic embryos in terms of morphological, histological, physiological, biochemical and genetic features. However, somatic embryos are more exposed to stress than their zygotic counterparts, accumulate less storage compounds and do not experience a growth arrest but germinate precociously (Winkelmann 2016).

Several proteins act as multifunctional regulators in both, zygotic and somatic embryogenesis. These include WUS, LEC1/LEC2, BBM1 and the AGAMOUS-LIKE 15 (AGL15) TFs (Fehér 2015). Not only the key regulators are common, but also the overall gene expression patterns of somatic and zygotic embryos are similar. When the cotton somatic and zygotic embryo transcriptomes were compared, the expression patterns of genes associated with metabolism, cellular processes and embryo development were found to be greatly similar (Jin et al. 2014). However, the main gene expression difference for in vitro cultured embryos resided within the stress-related gene class.

The study of regulatory molecules and connected gene networks during SE is of great significance for the long-term understanding of embryogenic competence and plant regeneration capacity, which is indispensable for crop improvement. While key role of miRNAs in zygotic embryogenesis was early demonstrated for *Arabidopsis thaliana* (Nodine and Bartel 2010; Armenta-Medina et al. 2017), their central function in somatic embryogenesis is starting to shape for different plant species (Chen et al. 2011; Wu et al. 2011; Zhang et al. 2012; Li et al. 2012; Lin et al. 2015; Yang et al. 2013; Chávez-Hernández et al. 2015; Zhang et al. 2017b).

12.6.2 Plant Regeneration Pathways

Based on the capability to regenerate whole plants from a variety of tissues or cells, such as leaf, pollen, root and endosperm cells, it is often claimed that all plant cells are totipotent. However, experimental data are scarce to sustain this statement. In vitro regeneration may progress through pre-existing stem cells in the plant body, and totipotency has been demonstrated only for certain, mostly young or partly differentiated tissues (Fehér 2015).

Also it has been suggested that dedifferentiation process includes the developmental switch of the explant cells to a pericycle cell-like functioning (Sugimoto et al. 2010). This implies that the early step in organogenesis involves cell redifferentiation to a distinct cell type, rather than to an 'undifferentiated/dedifferentiated' state (Horstman et al. 2017).

Somatic embryogenesis mainly follows two paths of regeneration depending on the developmental stage of the explant and culture conditions. That means somatic embryos can develop directly from the explant or indirectly from callus. The development of embryos is regularly indirect going through a pro-embryogenic cell mass (PEM) or embryogenic callus phase and only limited cells of the callus can form embryos (Fehér 2015). For example, cells that have undergone only a few divisions, such as asymmetrically dividing stem cells, can rapidly re-establish a removed stem cell niche of the root tip, the callus induced on Arabidopsis immature zygotic embryos can produce somatic embryos and the callus initiated from pericycle stem cells retains its ability to regenerate shoots.

For successful shoot regeneration from in vitro induced callus, it has been shown that lateral root primordial features are required and precede de novo shoot formation (Radhakrishnan et al. 2018). Morphology, cellular organization and molecular markers, such as WUSCHEL-related homeobox 5 (WOX5), SHORT-ROOT (SHR), SCARECROW (SCR), PLETHORA (PLT1/2), PIN1 and others, support the root identity of callus tissues (Sugimoto et al. 2010; Kareem et al. 2015). This is in accordance with the crucial role of auxin concentration in callus formation and further plant regeneration.

12.6.3 Pattern Formation During SE

During plant regeneration through SE, stem cells need cues to establish the conventional plant developmental patterning. Coordinated cell division and differentiation are required throughout plant regeneration to obtain a whole plant. Due to the existence of rigid walls limiting cell migration and rotation, pattern formation depends on positional information. Hence, the 'on-site' differentiation of newly formed cells comprises fundamental cell-to-cell communication. Molecules facilitating such events include peptides, phytohormones, transcription factors and small non-coding RNAs (Hisanaga et al. 2014).

The beauty of sRNA-mediated cell-to-cell signalling resides in avoiding the use of specific receptors and energy consuming sequential steps of signal transduction pathways preceding gene expression responses. Instead, it utilizes highly specific nucleotide base paring for direct suppression of target mRNA expression (Fig. 12.1). The mechanism underlying sRNA transference across the cell wall possibly involves plasmodesmata (PD).

12.6.3.1 sRNAs Involved in Shoot Apical Meristem Formation

Direct in vitro shoot regeneration is de novo committed by cytokinin (Radhakrishnan et al. 2018). This process is characterized by a clearance of epigenetic marks at the WUS locus. Upon transfer to cytokinin-rich medium, repressive histone mark H3K27me3 is gradually removed from the locus coincident with WUS expression at shoot regeneration sites. In *Arabidopsis thaliana*, WUS is expressed at the organizing centre (OC) located at the SAM inner stem cell layer (L3) and the corresponding protein moves to more external L2 and L1 layers to activate the production of CLV3, which eventually attenuates WUS expression (Schoof et al. 2000; Lee and Clark 2013). This feedback loop maintains the size of SAM stem cell pool constant, but does not explain how exactly the cellular organization operates in the context of stem cell division.

Recent reports have nicely demonstrated that both, CLV3 and B-type ARABIDOPSIS RESPONSE REGULATORs (ARRs) partnered by HD-ZIP III TFs, are required for WUS enhanced expression during shoot regeneration (Zhang et al. 2017a). Furthermore, regulation of HD-ZIP III TFs by miR165/166 restricts the regionalization of ARRs and miR394 acts as a positional cue by repressing the F-box protein LEAF CURLING RESPONSIVENESS (LCR) at the internal layer, where it interferes with CLV3 expression (Knauer et al. 2013). This repression allows stem cell maintenance and supports the precise interplay between cytokinin and auxin at the SAM.

Additional regulation by miR156 contributes to shoot regeneration potential. As Arabidopsis plants age, they lose their ability to regenerate shoots mostly due to reduced miR156 levels and up-regulation of its target SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) TFs (Zhang et al. 2015). Particularly, SPL9 directly interferes with the function of B-type ARRs, impairing the cytokinin response and consequently shoot regeneration. Plants overexpressing miR156 showed increased shoot regenerative ability and for longer periods. Interestingly, miR156 also declines during long-term callus subculture in maize (Dinkova and Alejandri-Ramirez 2014) and citrus (Long et al. 2018). In the last study, it was demonstrated that miR156 overexpression or the SPL9 orthologous gene knockdown rescued the embryogenic capacity of aged citrus callus supporting the central role of this miRNA-target module in SE regulation.

12.6.3.2 sRNAs Implicated in Root Apical Meristem Development

Similar to shoot regeneration, plant root regeneration is also guided by spatial complementary hormone domains (Efroni et al. 2016). However, low cytokinin:auxin ratios can promote root organogenesis, but not shoot regeneration. The root apical meristem (RAM) formation occurs in an auxin-dependent fashion during the early globular embryonic stage. The meristematic region is formed by stem cells organized around mitotically inactive cells called quiescent centre (QC). The root meristem size varies between species. While Arabidopsis has a small meristem with only four QC cells, maize RAM has 500–1000 cells (Jiang et al. 2010). Most studies on RAM establishment regulation have been done in Arabidopsis. Auxin polar transport from shoot to the root is carried out by PIN efflux transporters and generates auxin maximum at the root tip for expression of TFs at the stem cell niche, PLTs, SCR, SHR and WOX5 (Honkanen et al. 2017).

Impairing the sRNA biogenesis machinery (null *dcl1* mutant) displays early embryo patterning defects, including both SAM and RAM establishment (Nodine and Bartel 2010). Such defects mostly occur due to a precocious up-regulation of TFs that promote differentiation before the pluri-potential cell state can give rise to different cell types. Most of the miRNAs involved in auxin signalling (Fig. 12.3) are crucial for root tissue pattering. Particularly, miR165/166 are produced in the endodermis layer of root meristem, and move into other cell layers where HD-ZIP III TFs dose-dependent suppression is required for protoxylem and metaxylem specification (Carlsbecker et al. 2010).

12.6.3.3 sRNAs Involved in Tissue Polarity

The tasiRNA class was the first described mobile sRNAs acting in a cell non-autonomous manner. Particularly, tasiR-ARFs participate in leaf polarity by establishing opposite gradients for ARF3/4 and HD-ZIP III TFs (Chitwood et al. 2009). miR390 promotes tasiR-ARFs production at the adaxial layers of leaf primordia and they are spread in a gradient decreasing towards the abaxial side. ARF3/4 promote abaxial identity through positive regulation of miR165/miR166, which in turn represses HD-ZIP III TFs. This pathway is conserved in land plants and it is involved in the normal development of leaves, lateral roots and flowers. Moreover, HD-ZIP III mRNA accumulation, defined by miR165/miR166-dependent suppression, is restricted to the central-apical domain of globular stage embryos to promote SAM specification and to ensure cells at the basal pole to be correctly destined to root meristem (Smith and Long 2010). miR165/miR166 act cell non-autonomously from the basal part of the heart stage embryos and subsequently promote the apical fate at the subsequent stages (Miyashima et al. 2013). As additional control mechanism, AGO10 sequesters miR165/miR166 to protect HD-ZIP III mRNA (Zhu et al. 2011). Such regulation provides a novel mechanism by which the graded distribution of sRNAs is translated into an array of cell fates through a miRNA-dependent gene expression control.

12.6.4 sRNAs Abundance Switches in Somatic Embryo Development

sRNA role in the development of somatic embryos and plant regeneration is often overlooked, while significantly more attention has been paid to these molecules during the induction phase of SE. Most of the available research has focused in comparing gene expression programs at dedifferentiated status (i.e. embryogenic callus and or non-embryogenic callus) versus globular, heart and torpedo somatic embryos differentiating upon appropriate stimulus. During most recent years, global analyses using microarrays or next-generation sequencing (NGS) technology compared the presence of conserved and species-specific miRNAs at particular developmental stages of somatic embryos. However, most of the studies use pooled sRNA from the somatic embryos and embryogenic cultures to generate their library, so considerable information might be lost (Table 12.1).

Somatic embryo developmental stages have been established according to each species-specific plant regeneration method. While some species display distinguishable globular, heart, torpedo and cotyledonary embryos, others have assigned early, mid, late embryo or first, second, third stages in a temporary line starting from the differentiation induction. In spite of such heterogeneity, available sRNA data suggest that plant conserved miRNAs exert common functions during somatic embryo development (Fig. 12.2). With greater detail, we have summarized characteristic miRNA abundances reported at different developmental stages of plant regeneration for several species in Table 12.2. All data correspond to the analyses of

Table 12.1 Developmental stages of SE in different crops and type of analysed sRNAs

Specie	Developmental stage	Methods	sRNA	Reference
Oryza sativa (rice)	Differentiated callus	Northern blot NGS	miRNAs, tRNA-derived	Luo et al. (2006) Chen et al. (2011)
Citrus sinensis L. Osb. (orange)	Globular and cotyledon-shaped somatic embryo	qRT-PCR	miRNAs	Wu et al. (2011)
Larix leptolepis (larch)	Early, middle, late single embryo and cotyledonary embryo	NGS qRT-PCR	miRNAs	Zhang et al. (2012)
Liriodendron tulipifera (hybrid yellow poplar)	Stages of embryos: E5–E9	NGS Microarrays	miRNAs	Li et al. (2012)
Dimocarpus longan (longan)	Globular, torpedo-shaped, cotyledonary embryos	NGS, qRT-PCR	miRNAs tasiRNAs	Lin and Lai (2013) Lin et al. (2015)
Gossypium hirsutum (cotton)	Globular, torpedo, cotyledon-stage embryo	qRT-PCR	miRNAs	Yang et al. (2013)
Zea mays (maize)	First stage, second stage, plantlet	Northern blot qRT-PCR	miRNAs	Dinkova and Alejandri-Ramirez (2014) Chávez-Hernández et al. (2015)
Lilium pumilum DC. *Fisch.*	Globular, torpedo and cotyledon-stage embryos	NGS qRT-PCR	miRNAs	Zhang et al. (2017b)

Table 12.2 miRNA expression during somatic embryo differentiation in diverse plants

Different plant groups			Developmental stages of somatic embryo					
			Early stage		Middle stage		Late stage	
			Globular		*Heart*	*Torpedo*	*Cotyledonary*	
Angiosperms	Dicots	Orange[a]	miR156 miR159 miR164	miR390 miR397	miR394		miR166 miR167 miR398	
		Longan[b]	miR397a miR398b		miR159a,b,c,f miR160a miR398a,b	miR159a,b,c miR160a miR167a miR390a miR398b	miR156a miR159a,c miR167a	miR397a miR398b
		Cotton[c]	miR164 miR390				miR156 miR167 miR390	
		Lilium[d]	miR396 miR397 miR168 miR319			miR156 miR164 miR166 miR171	miR160 miR166 miR319	miR390 miR397 miR398 miR482
	Monocots	Maize[e]	*First stage*		*Second stage*		*Plantlet*	
			miR156 miR164 miR168 miR397	miR398 miR408 miR528	miR159 miR167 miR168	miR319 miR408 miR528	miR156 miR164 miR397 miR398	
		Rice[f]	miR150 miR156 miR157 miR158 miR159	miR164 miR166 miR167 miR169 miR394				

Different plant groups		Developmental stages of somatic embryo				
		Early stage	Middle stage		Late stage	
		Globular	*Heart*	*Torpedo*	*Cotyledonary*	
Gymnosperms	Larch[g]	*Early embryo*	*Middle embryo*		*Cotyledonary embryo*	
		miR162 miR168a, b miR171a,b,c	miR171a,b,c		miR159a,b,c miR160 miR162 miR166 miR167 miR168a,b	miR171a,b miR390 miR397 miR398

[a]Wu et al. (2011), Wu et al. (2015), and Long et al. (2018)
[b]Lin and Lai (2013), and Lin et al. (2015)
[c]Yang et al. (2013)
[d]Zhang et al. (2017b)
[e]Dinkova and Alejandri-Ramirez (2014), and Chávez-Hernández et al. (2015)
[f]Chen et al. (2011)
[g]Zhang et al. (2012)

bulk tissues composed mostly of heterogeneous cell types at each stage and might not reflect the precise miRNA expression switches. Recently, a new protocol was developed in *Arabidopsis thaliana* SE to visualize miRNA expression in a whole mount tissue using in situ hybridization (Wójcik et al. 2018). The application of such technique would be of utmost significance since, as discussed above, sRNA and target cell-specific distribution determines particular cell fates in tissues committed to the SE program.

For most plant species shown in Tables 12.1 and 12.2, early stages of differentiation are featured by the expression of miR159, miR164 and miR397. miR164 targets CUC2, a member of the plant-specific NAC domain (NAM, ATAF1/2 and CUC2) TF family, with important roles in plant development and stress responses (Aida et al. 1997). miR159 controls the transcript levels of MYB factors during seed germination and abiotic stress (Reyes and Chua 2007) and miR397, miR398 and miR408 regulate copper-dependent enzymes, such as superoxide dismutases (SOD) laccases and plantacyanin in response to copper deficiency (Abdel-Ghany and Pilon 2008; Sunkar et al. 2012).

It is well known that abiotic stress plays crucial role in modulating differentiation during SE. miR397 and miR398 are particularly abundant at early or late SE developmental stages for most of the analysed plant species. For example, miR398 increased during cotyledon-shaped embryo morphogenesis in orange and during formation of early staged embryo in larch. In *Dimocarpus longan* SE, miR398b, but not miR398a is highly expressed at heart-shaped and torpedo-shaped embryos. However, miR398b levels decreased during cotyledonary embryo development, leading to CSD accumulation and promoting embryo maturation (Lin and Lai 2013; Lin et al. 2015). For maize and rice, miR397, miR398, miR408 and the monocot-specific miR528 were present in both, dedifferentiated and differentiated tissues (Luo et al. 2006; Chen et al. 2011; Chávez-Hernández et al. 2015; Alejandri-Ramírez et al. 2018). Most of them were abundant at initial differentiation stages, but further decreased in the regenerated plantlet. Interestingly, laccases targeted by miR397 or miR528 have been associated with cell wall lignification and thickening during secondary cell growth (Constabel et al. 2000; Sun et al. 2018). Hence, miRNA-mediated down-regulation of laccases might associate with cell wall loosening in dedifferentiated tissues and early differentiation stages (Fig. 12.2).

The miR390-tasiR-ARF-ARF3/4 regulation also seems to operate in SE differentiation. miR390 was abundant at early globular-shaped embryo formation in *Citrus sinensis* (Wu et al. 2011) and *Gossypium hirsutum* SE (Yang et al. 2013), at heart and torpedo embryonic stages in *Dimocarpus longan* and in cotyledonary embryos for *Larix leptolepis* (Lin et al. 2015; Zhang et al. 2012). Correspondingly, *Dimocarpus longan* TAS3 and ARF4 exhibited their lowest expressions at the cotyledonary stage and reached their peaks in globular embryos. Interestingly, the miR390 primary transcript and TAS3 precursors were up-regulated by the synthetic auxin 2,4-D in a concentration-dependent manner (Lin et al. 2015).

Another miRNA participating downstream of auxin signalling, miR166, increased at later stages in *Citrus sinensis* cotyledon-shaped embryo morphogenesis (Wu et al. 2011, 2015), *Lilium pumilum* torpedo-shaped and cotyledonary embryos

(Zhang et al. 2017b) and *Larix leptolepis* cotyledonary embryos (Zhang et al. 2012). For *Dimocarpus longan* SE it was suggested that changes in miR166c* levels might be caused by alterations of endogenous gibberellin GA3 concentrations leading to the inhibition of early embryonic cell differentiation and globular embryo formation (Lin and Lai 2013). However, in *Oryza sativa*, miR166 increment was observed at early SE stages of differentiation (Chen et al. 2011).

The other two miRNAs that regulate ARFs, miR160 and miR167, would be expected to display auxin-dependent, tissue-specific expression patterns. Interestingly, miR160 was barely detectable at early, but highly expressed during heart- and torpedo-shaped embryonic stages of *Dimocarpus longan* SE (Table 12.2; Lin and Lai 2013). On the other hand, *Larix leptolepis* miR160 showed greater abundance at the cotyledonary embryo stage (Zhang et al. 2012). miR167 levels also increased during cotyledonary and mature embryonic stages for *Citrus sinensis*, *Dimocarpus longan*, *Gossypium hirsutum* and *Larix leptolepis* (Wu et al. 2011; Zhang et al. 2012; Yang et al. 2013; Lin et al. 2015). However, this miRNA showed contrasting behaviour in SE depending on the plant species and in vitro culture conditions. For example, rice miR167 decreased when cells, cultured in the presence of auxins, were transferred to an auxin-free medium (Yang et al. 2006), whereas Longan miR167 was undetectable in a medium containing 2,4-D (Lin and Lai 2013). In cotton and maize, miR167 also exhibited up-regulation in the dedifferentiated tissues (Yang et al. 2013; Alejandri-Ramírez et al. 2018). Whether miR167 participates in the SE process through regulating its targets ARF6/8 in response to external auxin levels remains to be elucidated.

As previously mentioned, miR156 mostly regulates tissue embryogenic potential through its SPL targets. It is required at early zygotic embryogenesis (Nodine and Bartel 2010) and during early SE (Long et al. 2018). However, its levels also progressively increased at later differentiation stages for cotton, Longan and maize SE (Table 12.2). A perfect inverse expression pattern was found for the SPL transcript and miR156 during cotton embryo development (Yang et al. 2013). On the other hand, in maize plant regeneration, miR156 also showed initial increase coincident with its target reduction during the differentiation process (Chávez-Hernández et al. 2015). Such behaviour supports its central role in SE for different plant species.

12.7 Conclusions and Perspectives

SE is a noteworthy model to study early developmental features of embryogenesis, molecular aspects of cell differentiation, and is a powerful tool for plant biotechnology. Exploring the role of different sRNA classes in this process constitutes a promising tool to understand the basis of totipotency as well as to achieve successful plant regeneration through the process. Recent progress of sRNA research in agricultural plants has been emphasized on trait regulation, stress responses and reproduction. Taking into account that SE covers a response of the plant to stressful conditions aiming to preserve its potential to further grow and reproduce in the

future, it represents a unique system to challenge our knowledge on developmental molecular cues. Although *Arabidopsis thaliana* has been a great model for sRNA pathways dissection, it urges to extend this research to diverse economically relevant plants. In this sense, SE represents an excellent model to understand sRNA cell-specific fate, target regulation, responses to phytoregulators, stress and differentiation stages. Further exploration of particular to SE sRNA regulatory nodes would provide insights into the development of appropriate tools for crop improvement.

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