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FOCUSED REVIEW

Keep calm and carry on: miRNA biogenesis under stress

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SUMMARY

MicroRNAs (miRNAs) are major post-transcriptional regulators of gene expression. Their biogenesis relies on the cleavage of longer precursors by a nuclear localized processing machinery. The evolutionary preference of plant miRNAs to silence transcription factors turned these small molecules into key actors during growth and adaptive responses. Furthermore, during their life cycle plants are subject to changes in the environmental conditions surrounding them. In order to face these changes, plants display unique adaptive capacities based on an enormous developmental plasticity, where miRNAs play central roles. Many individual miRNAs have been shown to modulate the plant response to different environmental cues and stresses. In the last few years, increasing evidence has shown that not only individual genes encoding miRNAs but also the miRNA pathway as a whole is subject to regulation in response to external stimulus. In this review, we discuss the current knowledge about the miRNA pathway. We dissect the pathway to analyze the events leading to the generation of these small RNAs and emphasize the regulation of core components of the miRNA biogenesis machinery.

Keywords: microRNAs, stress response, biogenesis.

INTRODUCTION

Small RNAs are essential regulators of gene expression in plants and animals. They can be classified in different groups according to their biogenesis pathways (reviewed in Axtell, 2013; Bologna and Voinnet, 2014). MicroRNAs (miRNAs), small RNAs of 20–22 nt in length, control plant development, growth, stress adaptation and other physiological processes by regulating the expression of many transcription factors and stress-responsive proteins (Li *et al.*, 2017). This characteristic makes miRNAs master regulators of the plant response to the surrounding environment. Like many other genes in the plants, multiple individual miRNAs are regulated under given environmental or developmental conditions to ensure physiological homeostasis during plant growth and in responses to external stimuli. Nevertheless, not only individual miRNAs respond to changes in the environment. In the past few years, many reports have presented evidence indicating that the miRNA processing and biogenesis machinery is tightly controlled by external signals. This implies that plants can shut down, or activate, simultaneously the production of numerous miRNA families with massive regulatory consequences. In this review, we discuss the current knowledge about miRNA biogenesis and how it can be modulated in response to external signals.

MICRORNA PROCESSING IN PLANTS

MicroRNAs (miRNAs) are distinguished from other small RNAs by their precise excision from a larger precursor harboring an imperfect stem-loop structure (Axtell, 2013; Bologna and Voinnet, 2014). In plants, DICER-LIKE 1 (DCL1) and accessory proteins such as hyponastic leaves 1 (HYL1) and serrate (SE) process these precursors to release the actual miRNAs, which are in turn loaded to an argonaute (AGO) protein, usually AGO1. Mature miRNAs of around 21 nt pair to longer RNAs and guide them to cleavage or translational arrest (reviewed in Rogers and Chen, 2013; Yu *et al.*, 2017). Many evolutionarily conserved miRNAs regulate transcription factors involved in plant development and hormone signaling, and thus impairing the function of general components of the miRNA pathway causes strong developmental and growth defects.

Box 1 Bullet-point summary

- Plant miRNA precursors are processed in different ways
- MiRNA biogenesis can be activated or repressed in response to external stimuli
- The post-translational regulations of miRNA-biogenetic components modulate miRNA biogenesis

Genes coding for miRNAs (*MIRNAs*) are transcribed by RNA polymerase II (Pol II) as longer transcripts (pri-miRNA) that are capped and polyadenylated. In certain cases, pri-miRNAs are also spliced, and the presence of introns downstream of the miRNA precursor can stimulate its processing (Bielewicz *et al.*, 2013; Schwab *et al.*, 2013). As in protein-coding genes, the transcription of MIRNAs is under the control of specific transcription factors that in turn respond to developmental cues or changes in the environment. There are some examples of characterized miRNA precursors located in introns of protein-coding genes, such as miR400 (Yan *et al.*, 2012) and miR402 (Knop *et al.*, 2016); however, in phylogenetic analyses these intronic miRNAs are only present in *Brassicacea* (Fahlgren *et al.*, 2010), whereas evolutionarily conserved miRNAs are encoded by independent transcriptional units.

Recent studies suggest that at least part of the miRNA processing machinery is recruited to the pri-miRNAs during transcription. The Elongator complex is a general regulator of miRNA expression. Being able to interact with both Pol II and DCL1, the Elongator complex might facilitate the establishment of a processing complex during transcription (Fang *et al.*, 2015a). Similar functions have been attributed to components of the MOS4-associated complex (Zhang *et al.*, 2013, 2014a; Jia *et al.*, 2017; Li *et al.*, 2018), a general factor that associates with the spliceosome and is conserved in plants and animals (Koncz *et al.*, 2012). After these initial events of transcription and recruitment of factors to the nascent primary transcript, the miRNA processing machinery needs to cut the stem-loop precursor to release the small RNA. DCL1, a type-III Ribonuclease, will perform all cuts in the precursor and release the miRNA (Figure 1; Park *et al.*, 2002; Reinhart *et al.*, 2002; Kurihara and Watanabe, 2004). A few exceptions include some recently evolved miRNAs such as miR822 and miR839, which are processed by DCL4 (Rajagopalan *et al.*, 2006).

Many proteins have been identified that participate in the processing of pri-miRNAs (recently reviewed in Achkar *et al.*, 2016; Yu *et al.*, 2017); however, the core components are formed by DCL1, together with the dsRNA-binding protein HYL1 (Han *et al.*, 2004; Vazquez *et al.*, 2004) and the zinc-finger protein SE (Lobbes *et al.*, 2006; Yang *et al.*, 2006). *In vitro* reconstitution experiments have shown that these proteins are sufficient to accurately process miRNA precursors (Dong *et al.*, 2008; Zhu *et al.*, 2013). DCL1 and HYL1 interact with each other and co-localize in nuclear speckles, termed 'dicing bodies' (Fang and Spector, 2007). The processing of the miRNA precursors might occur co-transcriptionally or, alternatively, in dicing bodies after an initial recruitment of processing factors during

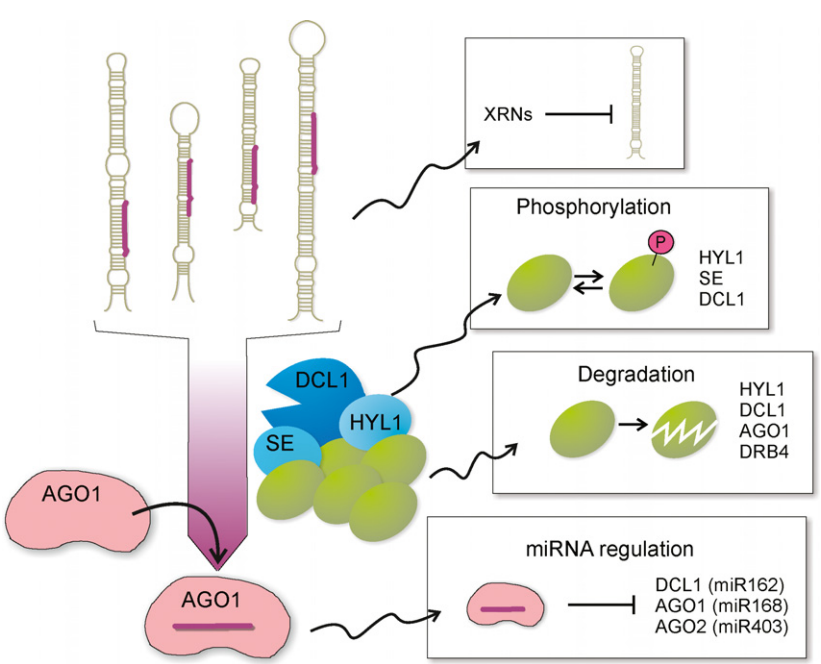

Figure 1. Scheme showing the miRNA biogenesis pathway and regulatory steps. Precursors with many different structures are processed by a multiprotein complex that harbors DCL1, HYL1 and SE as core components. This complex cuts the precursors to release the miRNA that becomes incorporated in an AGO protein, generally AGO1. Regulatory steps include the control of XRN activity, protein phosphorylation/dephosphorylation, protein stability and feedback regulation by miRNAs targeting DCL1 and AGO1.

transcription. Future work will uncover the dynamics and subcellular localization of the different steps in miRNA biogenesis.

After cutting the pri-miRNA, the processing machinery releases a double-stranded miRNA of approximately 21 nt with 2-nt 3′ overhangs (an miRNA/miRNA* duplex). This molecule is loaded to an AGO protein, generally AGO1, with the aid of HFSP90 (Iki *et al.*, 2010; Zhang *et al.*, 2014b; Bologna *et al.*, 2018). AGO1 then retains one strand and releases the other, commonly the miRNA*. In given situations or tissues, however, the miRNA* can also be retained and have regulatory functions (Zhang *et al.*, 2011; Manavella *et al.*, 2013). The mechanisms for strand selection of miRNA over miRNA* and AGO loading are not yet fully understood in plants; however, the structure of the duplex and the identity of the 5′ base (reviewed in Fang and Qi, 2016), as well as HYL1 (Eamens *et al.*, 2009), have been shown to be important for the selection of the miRNA strand. It has been recently proposed that empty AGO1 is localized in the nucleus, but after loading of the miRNA a conformational change exposes a nuclear export signal, so that the AGO1-miRNA complex moves to the cytoplasm where it exerts its function (Bologna *et al.*, 2018). Thus, all steps of miRNA biogenesis seem to occur in the plant nucleus.

Given that plant miRNA precursors are of variable size and shape, an important question is how pri-miRNAs are distinguished from the many other hairpin-containing transcripts for processing by the DCL1 complex. At least part of the specificity during miRNA biogenesis is achieved through structural determinants present in the miRNA precursors. A large group of miRNA precursors harbor a dsRNA stem of 15–17 bp below the miRNA/miRNA* duplex and above an internal bubble (Mateos *et al.*, 2010; Song *et al.*, 2010; Werner *et al.*, 2010; Bologna *et al.*, 2013; Zhu *et al.*, 2013). This structural feature is recognized by the processing complex to produce a first cut, establishing one end of the miRNA and releasing a hairpin from the initial transcript (Song *et al.*, 2010; Werner *et al.*, 2010; Bologna *et al.*, 2013; Zhu *et al.*, 2013). Although mechanistic aspects of this recognition process remain to be elucidated, evidence has been accumulated in animals that might be extrapolated to plant systems. The first cut in animal miRNA biogenesis is performed by Drosha, which has a similar structure to Dicer (Nguyen *et al.*, 2015; Kwon *et al.*, 2016). Drosha acts as a molecular ruler recognizing the transition from single-stranded RNA (ssRNA) to double-stranded RNA (dsRNA), producing a cut 11 nt away from this junction (Nguyen *et al.*, 2015; Kwon *et al.*, 2016). This mechanism might be similar to that of DCL1 in plants, albeit with a difference in the distance where both enzymes catalyze the miRNA precursor cut.

Yet plant miRNA precursors can be processed in additional modes. A group of plant precursors have a dsRNA region of 15–17 bp above the miRNA/miRNA (Bologna *et al.*, 2013; Chorostecki *et al.*, 2017). In this case, a first cut by DCL1 is produced in the distal part of the precursor, below a small terminal loop, and the precursor processing proceeds in a loop-to-base direction (Addo-Quaye *et al.*, 2009; Bologna *et al.*, 2009, 2013; Moro *et al.*, 2018). In either case, after the first cleavage reaction, DCL1 produces a second cut ~21 nt away from the first cut releasing the miRNA/miRNA* duplex. Certain precursors, which have a long dsRNA structure, are processed sequentially by three or four DCL1 cuts every ~21 nt, generating several small RNA duplexes (Kurihara and Watanabe, 2004; Addo-Quaye *et al.*, 2009; Bologna *et al.*, 2009; Zhang *et al.*, 2010). These different miRNA biogenesis modes highlight the plasticity of plant miRNA biogenesis. Furthermore, the structural determinants present in the precursors that guide the processing machinery are conserved during evolution (Chorostecki *et al.*, 2017; Xia *et al.*, 2017).

In certain cases, structural elements present in the pri-miRNAs can dampen the production of the miRNA. Precursors that harbor a terminal branched loop can be recognized by the DCL1 processing complex to generate unproductive cuts (Zhu *et al.*, 2013). Under normal conditions the miRNA-processing complex in plants is rather accurate in most cases (Moro *et al.*, 2018); however, in plants deficient in HYL1 or SE, cryptic determinants might be recognized in the precursors leading to aberrant cuts (Moro *et al.*, 2018), suggesting that these cofactors also help DCL1 to identify the correct processing determinant. BRAHMA is the ATPase subunit of the large switch/sucrose non-fermentable (SWI/SNF) complex, known by its broad role in the regulation of chromatin structure and transcription (reviewed in Clapier *et al.*, 2017). Recent studies have shown that BRAHMA interacts with SE and is able to alter the secondary structure of the miRNA primary transcripts, thus inhibiting their processing (Wang *et al.*, 2018). Interestingly, the interaction between BRAHMA and SE is required to modify the structure of the miRNA precursors, but it is not necessary for the transcriptional activity of BRAHMA (Wang *et al.*, 2018).

Although miRNA processing is expected to be accurate, in certain cases the flexibility in the generation of small RNAs might provide novel functions. miR168 is an evolutionarily conserved miRNA that regulates AGO1, therefore conferring feedback regulation of the miRNA pathway (Vaucheret *et al.*, 2006). The miR168/miR168* duplex has a flexible central region that allows three alternatives for base-pairing between the two RNA strands (Iki *et al.*, 2018). Processing of the precursors with different conformation will lead to mature miR168 with different sequences and properties, including a 22-nt miR168 isoform that is preferentially loaded to AGO10 instead of AGO1 (Vaucheret, 2009; Iki *et al.*, 2018). The 22-nt miR168-AGO10 triggers the generation of secondary siRNAs from AGO1, amplifying

the repression of AGO1 (Iki *et al.*, 2018). From a global perspective, it has been noticed that precursors processed from the loop tend to generate more variable miRNAs than those processed from the base (Moro *et al.*, 2018). Interestingly, several proteins have been found to bind to specific miRNA precursors in animals, stimulating or inhibiting the biogenesis of a given miRNA (Michlewski and Caceres, 2019). Such a scenario has not yet been observed in plants. Instead, miRNA biogenesis seems to be integrally regulated in several different ways, from miRNAs targeting core components of the miRNA pathway to signaling cascades that regulate the activity and stability of components of the miRNA pathway (Figure 1).

ENVIRONMENTAL REGULATION OF THE *MIRNA* BIOGENESIS MACHINERY

A quick and effective response to environmental stimuli is essential for the proper acclimation of plants as sessile organisms. In this sense, many miRNAs have fundamental roles during the adaptation of plants to most biotic and abiotic stresses (Kumar, 2014; Megha *et al.*, 2018). In contrast to *MIRNA* genes that are transcriptionally regulated under stressful conditions, thus modifying the levels of the encoded miRNAs, the miRNA processing machinery was long believed to be ubiquitous and invariable in plants. In recent years, however, it has become clear that the production of mature miRNAs from pri-miRNAs is highly regulated both in a tissue/developmental-stage manner and by environmental cues.

Post-translational modifications have emerged as a key regulatory component of the core miRNA-biogenesis machinery (regulatory processes are discussed further in the next section). In this sense, the phosphorylation of DCL1 was shown to be a requirement for its interaction with DAWDLE (Machida and Yuan, 2013), which in turn is necessary for proper DCL1 activity (Zhang *et al.*, 2018). HYL1 activity, essential for DCL1 accurate processing, is also controlled by its phosphorylation state. CPL1/2 and PP4 were shown to de-phosphorylate HYL1, thereby making it active (Manavella *et al.*, 2012; Su *et al.*, 2017). Conversely, MPK3 and SnRK2 are able to phosphorylate this protein, thereby inactivating it (Raghuram *et al.*, 2015; Yan *et al.*, 2017). SnRK2s, activated in response to ABA and other environmental stresses, also phosphorylate SE, suggesting that HYL1 and SE activity could be modulated under stress by phosphorylation (Yan *et al.*, 2017). Besides making the protein inactive, HYL1 phosphorylation restricts the protein subcellular localization to the nucleus, protecting it from proteolytic degradation triggered during stressful periods of limited light (Cho *et al.*, 2014; Achkar *et al.*, 2018). Regulator of CBF Gene Expression 3 (RCF3, also known as HOS5 or SHINY), the expression of which is reduced by salt, hyperosmotic stress and ABA, assists CPL1 during HYL1 de-phosphorylation in a tissue-specific manner (Guan *et al.*, 2013; Jeong *et al.*, 2013; Jiang *et al.*, 2013; Chen *et al.*, 2015; Karlsson *et al.*, 2015). CPL1 and SnRK2s can also alter the phosphorylation status of SE and RCF3 with still unclear physiological and molecular effects (Chen *et al.*, 2013, 2015; Wang *et al.*, 2013; Yan *et al.*, 2017). A similar uncertainty holds true for the physiological role of DCL1 phosphorylation; however, as one of the most influential environmental cues for plants, light was shown to affect not only the stability of HYL1 but also the stability of DCL1. The basic helix-loop-helix (bHLH) transcription factor phytochrome-interacting factor 4 (PIF4) interacts with DCL1 and HYL1, destabilizing these proteins during the transition from dark to red light in a process that is not yet fully understood (Sun *et al.*, 2018).

Interestingly, these reports imply that under given environmental conditions the whole miRNA processing machinery could be turned down, leading to a general reduction in the miRNA population. In such a scenario, the steady levels of each mature miRNA, their molecular stability and turnover rate could drastically impact which genes remain under miRNA regulation after DCL1/HYL1 degradation, and for how long. Along with this idea, the expression of HEN1, which methylates and protects miRNAs from degradation, is highly regulated by light, which would directly impair the mature miRNA pool and thus the impact of DCL1/HYL1 degradation in such conditions (Tsai *et al.*, 2014). A balance between miRNA production and stability would be expected to govern the miRNA regulatory network under different light conditions. In a beautiful example of environmentally controlled miRNA turnover, Fang and colleges reported late last year that under heat, a retrograde signaling from the chloroplast, through vitamin E and 3′-phosphoadenosine 5′-phosphate, inhibits nuclear exoribonucleases (XRNs) protecting pri-miRNAs and thus promoting miRNA production (Fang *et al.*, 2018). The rice nucleotide transferase OsNTP3, the closest homolog to Arabidopsis HESO1 that uridylates and triggers the degradation of unmethylated miRNAs, is strongly reduced by cold but is enhanced by ABA, allowing us to envision another scenario where the environment controls miRNA stability (Ren *et al.*, 2014; Tu *et al.*, 2015; Yang *et al.*, 2017). Besides this potential change in miRNA stability under cold, it was reported that a reduction in ambient temperature enhances the processing of some miRNAs, even in the absence of the key DCL1 cofactors such as HYL1 and SE (Re *et al.*, 2019). In combination, these reports suggest that the repertoire of miRNAs acting in a given natural environment, where light, temperature, and stressful conditions are variable, will not only depend on each *MIRNA* transcription but also on the availability of miRNA processing factors, miRNA and pri-miRNA stability, as well as potential alternative processing pathways.

In the last years many cofactors controlling and fine-tuning miRNA production were identified using genetic

screening (Achkar *et al.*,2016). Different from core miRNA processing components, such as DCL1, SE and HYL1, the mutations of which produce embryonic lethality or severe developmental abnormalities, the absence of most of these newly identified cofactors only impact miRNA production at moderate levels and/or in given circumstances/tissues. Even when most of these cofactors are considered regulatory/accessory proteins to the core miRNA-processing complex, little is known about their expression patterns and regulation under stress. Most of these proteins have been characterized in the miRNA pathway under control conditions. Database mining suggested that several miRNA cofactors are regulated under stressful conditions (Figure 2); however, further experimental analyses are necessary to confirm where and under which conditions these genes are expressed, and the role that they play in such circumstances. Nevertheless, evidence is accumulating showing that the environment controls several of these factors. The cycling DOF transcription factor (CDF2), which binds miRNA promoters and interacts with DCL1 to regulate the accumulation of some miRNAs, follows a circadian rhythm of expression controlling the photoperiodic flowering response (Fornara *et al.*, 2009; Sun *et al.*, 2015). STABILIZED 1 (STA1), which has been suggested to control pri-miRNA splicing and to modulate DCL1 transcription, is particularly highly expressed when plants are under cold stress (Lee *et al.*, 2006; Ben Chaabane *et al.*, 2013). Similarly, SICKLE (SIC), which participates in intron splicing and miRNA biogenesis as a cofactor, is also involved in the response to cold temperatures (Fornara *et al.*, 2009; Sun *et al.*, 2015). The stress-related SWR1 Chromatin Remodeling Complex was recently associated with control over miRNA levels. In this particular case the regulation of miRNA levels by SWR1 is the consequence of a direct transcriptional regulation of miRNA genes rather than their processing (Choi *et al.*, 2016). Environmental cues are also expected to regulate Dicer-Like genes given the transcriptional control that CMA33/XCT, a protein previously shown to respond to light and hormone signaling, has over them (Martin-Tryon and Harmer, 2008; Ellison *et al.*, 2011; Fang *et al.*, 2015b). The Nuclear Cap-Binding Complex, which affects many RNA-related processes, including miRNA biogenesis, also responds to a wide variety of environmental conditions, processes recently reviewed by Daszkowska-Golec (2018). The proteins of the MOS4-associated complex (MAC) CDC5, PRL1 and MAC7, which interact with DCL1/SE, pri-miRNAs and HYL1, respectively, are responsive to different stresses where they have important roles (Zhang *et al.*, 2013, 2014a; Jia *et al.*, 2017). Interestingly, most of these cofactors only seem to affect subpopulations of miRNAs. Based on our current knowledge about how these cofactors respond to stresses we can envision a scenario in which a plant will have a particular repertoire of mature miRNAs specified by the combination of cofactors expressed in each tissue and under specific ambient conditions.

Even when an miRNA is produced in a certain tissue it may still not be active, however. AGO1, as the main component of the miRNA effector complex, also responds to the environment, rendering its activity variable. In this sense, AGO1 was shown to participate in the response to several stresses, including UV-induced DNA damage, salt stress and hormone responses (Dolata *et al.*, 2016; Schalk *et al.*, 2017; Liu *et al.*, 2018). In addition, some of the few proteins known to interact with AGO1 in plants are also heavily regulated by stress. HSP40/70/90, essential chaperons for the RISC assembly and AGO1 membrane association, are regulated under stressful conditions, potentially limiting the activity of the pathway under certain conditions (Barghetti *et al.*, 2017; Sjogren *et al.*, 2018). In the same way, the stress-responsive ubiquitin E3 ligase HOS1 controls AGO1 levels by regulating miR168 transcription, which in turn is able to target and silence *AGO1* mRNA (Wang *et al.*, 2015).

PROTEOLYTIC REGULATION OF AGO1, DCL1, HYL1 AND DRB4

Considering the importance of AGO1 in gene-silencing mechanisms, it is not surprising that AGO1 homeostasis is tightly regulated during plant development, growth and defense. The AGO1 transcript is itself a target of miR168 and AGO1-catalyzed mRNA cleavage (Vaucheret *et al.*, 2006). In the self-regulation pathway, the transcriptional co-regulation of *MIR168* and *AGO1* and post-transcriptional stabilization of miR168 by AGO1 are also involved in modulating how efficiently RISC complexes are assembled (Vaucheret *et al.*, 2006; Vaucheret, 2008). Plants use the self-regulatory processes of AGO1 to fine-tune the balance of miRNAs and siRNAs, and their target mRNAs, to adjust their development and growth (Vaucheret, 2008; Martínez de Alba *et al.*, 2011).

In general, proteolytic pathways, including proteases, the ubiquitin–proteasome system (UPS) and autophagy regulate the homeostasis of many proteins (Baker *et al.*, 2011; Bustamante *et al.*, 2014). Therefore, in addition to post-transcriptional regulation, AGO1 homeostatic regulation by proteolysis could play a role. The first evidence of AGO1 degradation was found in the context of plant–virus interactions. Many viruses use viral suppressors to neutralize the AGO1-dependent defense system of plants. For instance, the cucumber mosaic virus (CMV)-encoded 2b protein directly associates with AGO1 to inhibit the AGO1-mediated defense system (Zhang *et al.*, 2006). Recent studies have revealed that several viral suppressors function in the proteolytic regulation of AGO1. Polerovirus-encoded F-box protein (P0), a viral suppressor, targets the PAZ motif of AGO1 and mediates its degradation (Baumberger *et al.*, 2007). F-box proteins form part of the SCF Skp1-Cullin-F-box protein (SCF) E3 ubiquitin ligase complexes, which

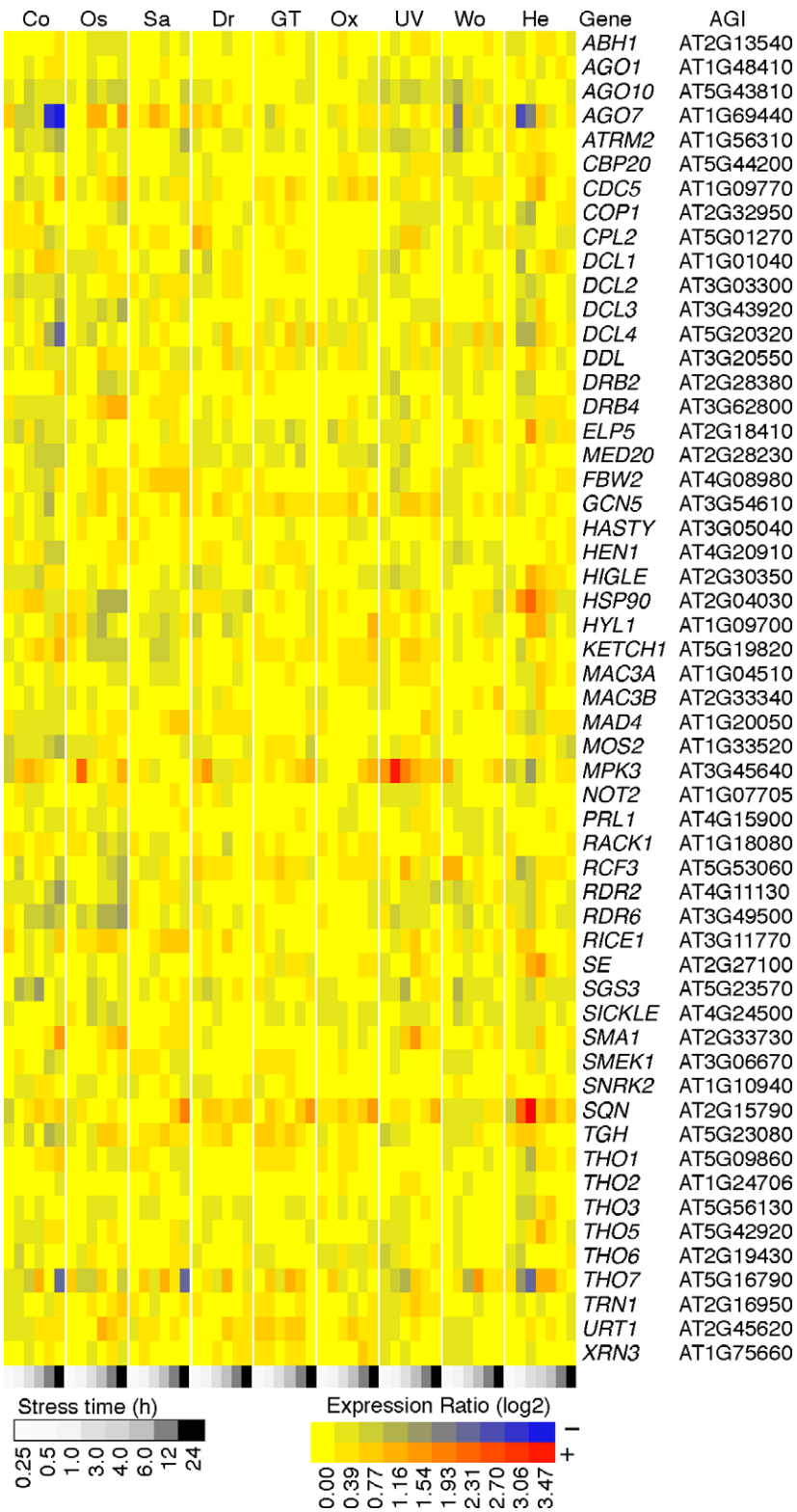

Figure 2. miRNA-related gene expression under stress. Expression of miRNA-related genes under stress conditions using the electronic Northern analysis tool of the Bio-Analytic Resource for Plant Biology web server. The heat map shows expression rates (log2) in plants subjected to cold (Co), osmotic (Os), salt (Sa), drought (Dr), genotoxic (GT), oxidative (Ox), UV-B (UV), wounding (Wo) and heat (He) stress, compared with control Arabidopsis shoots, for different periods of time.

tether poly-ubiquitins to target proteins for UPS-mediated degradation (Hare *et al.*, 2003; Cardozo and Pagano, 2004; Callis, 2014). Hence, by mimicking the host F-box protein, viral F-box proteins (e.g. P0) can sequester the host SCF ubiquitin–protein ligase (E3) system to disable the host defense system. The hijacking ability of P0 has been

demonstrated for Cucurbit aphid-borne yellows virus (CABYV) (Pazhouhandeh *et al.*, 2006). The targeted degradation of AGO1 by P0 was insensitive to the inhibition of the *26S* proteasome, however, implying that AGO1-P0 is not degraded by the UPS pathway (Baumberger *et al.*, 2007). This contradictory phenomenon – AGO1 stability is regulated by viral F-box proteins but is not degraded by the UPS – was verified in later studies. The F-box WD-repeat domain-containing protein 2 (FBW2) is known to mediate the ubiquitination of F-box interacting targets. In the absence of FBW2, AGO1 accumulated to a high level, and the overexpression of FBW2 notably decreased the level of AGO1, demonstrating that FBW2 is a negative regulator of AGO1 stability. The reduced level of AGO1 in FBW2-overexpressing transgenic plants was insensitive to treatment with the proteasome inhibitor MG132, however (Earley *et al.*, 2010). The enamovirus P0 suppressor can inhibit local and systemic RNA-silencing systems by destabilizing AGO1 in a similar way to polerovirus P0 (Fusaro *et al.*, 2012). Likewise, ectopic expression of P0 in Arabidopsis led to the degradation of AGO1 and this was also unaffected by the inhibition of the *26S* proteasome (Derrien *et al.*, 2012).

A study on the involvement of the autophagy pathway suggested a solution to the contradictory phenomenon in P0-mediated AGO1 degradation. Under the ectopic expression of P0 in Arabidopsis, AGO1 dramatically accumulated in response to treatment with 3-MA, an autophagy flux inhibitor, or E64, an autophagic protease inhibitor. Furthermore, AGO1 co-localized with ATG8, an essential ubiquitin-like protein that covalently attaches to the membrane of the autophagosome, strongly indicating that AGO1 is possibly associated with the autophagosome (Derrien *et al.*, 2012). Selective autophagy regulates specific cellular components via autophagy receptors that recognize signals, including ubiquitin, that target protein complexes, aggregates and whole organelles for selective autophagy (Kraft *et al.*, 2010; Myeku and Figueiredo-Pereira, 2011). In this context, the autophagy-dependent degradation of ubiquitinated AGO1 (Derrien *et al.*, 2012) would be connected with host SCF E3 ligase complexes, which are snatched by the P0 suppressor. Other viral suppressors do not always promote the autophagy-dependent degradation of AGO1, however. For instance, P25 is the viral suppressor of Potato virus X, which directly interacts with AGO1. When MG132 was infused into *Nicotiana benthamiana* leaves 2 h before co-infiltration with *Agrobacterium* strains to express P25 and AGO1, the level of AGO1 increased approximately 10-fold, even in the presence of the P25 suppressor, suggesting that AGO1 destabilization can be modulated by the *26S* proteasome (Chiu *et al.*, 2010). The two plausible pathways for AGO1 destabilization, the autophagic and UPS pathways, could function independently in plants according to the type of viral suppressor. For instance, P0 directs AGO1 to autophagy, whereas P25 guides AGO1 to UPS-mediated degradation; however, MG132 is not a specific inhibitor for the *26S* proteasome and can inhibit many serine-threonine proteases (Steinhilb *et al.*, 2001; Kisselev *et al.*, 2012). Therefore, pathways in a non-viral context, in addition to the two independent and pathogen-specific pathways, should be further investigated for AGO1 degradation using specific inhibitors and mutants for each proteolytic pathway, such as autophagy, UPS and other proteases (Figure 3a–c).

A recent study demonstrated that constitutive photomorphogenic 1 (COP1), a suppressor of photomorphogenesis, plays a crucial role in miRNA biogenesis. COP1 was essential for the proteolytic stabilization of HYL1. Deficiency of COP1 led to the reduction of HYL1 that subsequently compromised miRNA biogenesis. The levels of HYL1 were increased by MG132 treatment but not by the treatment of other specific proteasome inhibitors: clasto-lactacystin β-lactone, epoxomicin and PRY-41, an inhibitor of the E1 enzyme that functions in the ubiquitin-conjugating process. These results showed that UPS is not responsible for HYL1 degradation. In addition, the possible involvement of autophagy was investigated using the autophagy inhibitor 3-MA and the activator BTH, and HYL1 was found not to be degraded by autophagy. Moreover, HYL1 was specifically cleaved into a ~26-kDa N-terminal fragment by a crude cytoplasmic extract *in vitro*. The cleavage of HYL1 was efficiently blocked by either MG132 or E64 *in vitro* and *in vivo*. Based on these results, it was suggested that HYL1 might be cleaved by an as yet unknown protease that exists in the cytoplasm (Figure 3d). Furthermore, light is essential for the stability of HYL1. During the daytime, COP1 moves to the cytoplasm to stabilize HYL1, possibly by inhibiting the unknown protease. In contrast, during the night, COP1 returns to the nucleus, and that releases the protease activity required for HYL1 degradation (Figure 3e). These results indicate that light signaling integrates into miRNA biogenesis. In contradiction to this study, Sun *et al.* (2018) suggested that red light induces the destabilization of DCL1 and HYL1 by unknown proteases. In the study, DCL1 and HYL1 accumulated in 4-day-old etiolated seedlings and were rapidly degraded by irradiation for 3 h with red light. To reconcile this discrepancy between the two studies – the former study showed light-mediated stabilization of HYL1, whereas the latter study showed red light-induced degradation of DCL1 and HYL1 – detailed investigations using light signaling mutants under various light-treatment conditions need to be undertaken. Despite the discrepancy, these studies showed that a third proteolytic regulatory mechanism exists, distinct from both the UPS pathway and autophagy in the miRNA-biogenetic pathway.

Phosphorylation is one of the factors that determine the half-life of a protein. Yan *et al.* (2017) suggested that SnRK2 kinases directly phosphorylate HYL1, and that this

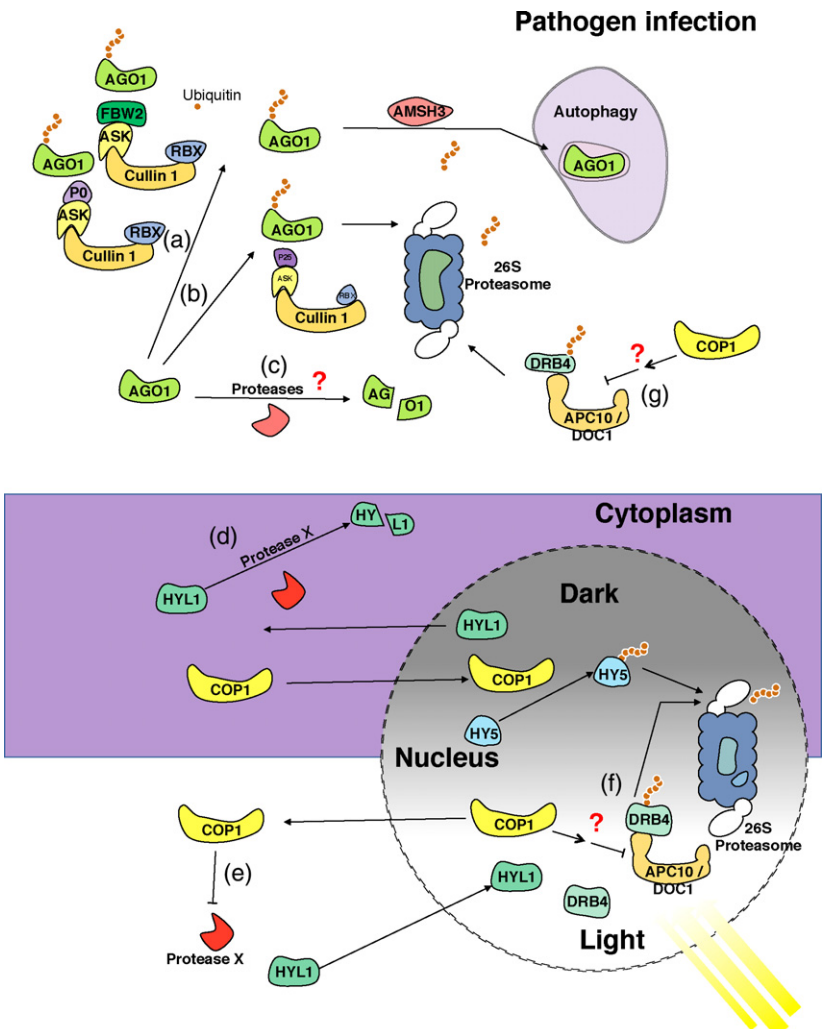

Figure 3. Proteolytic regulation of miRNA-biogenetic components. (a) AGO1 is kidnapped by P0 and FBW2 for selective autophagy-mediated degradation. (b) AGO1 is degraded by the *26S* proteasome after P25-Cullin complex-mediated ubiquitination. (c) A possible degradation of AGO1 by an unknown protease. (d) HYL1 is cleaved by an unknown protease X in the cytoplasm during the night. (e) COP1 E3 ligase inhibits the functionality of the unknown protease X, and therefore HYL1 safely moves into the nucleus during the daytime. (f) DRB4 is a potential target of the nuclear APC10/DCP1 E3 ligase complex. (g) Viral infection promotes the cytoplasmic localization of DRB4. DRB4 is possibly degraded by the APC10/DCP1 E3 ligase complex that can be protected by COP1 E3 ligase in the cytoplasm.

is required for the maintenance of the steady-state level of HYL1. Deficiency of *SnRK2* led to a notable reduction in HYL1 in rosette leaves, suggesting that non-phosphorylated HYL1 can be unstable. In contrast, Protein Phosphatase 4 (PP4) and the Suppressor of MEK 1 (SMEK1) complex dephosphorylate HYL1 to promote miRNA biogenesis by antagonizing the MAPK cascade in Arabidopsis. Deficiency of SMEK1 accelerates the degradation of HYL1, suggesting that phosphorylated HYL1 is more unstable than its unphosphorylated form (Su *et al.*, 2017). Achkar *et al.* (2018) explained that the discrepancy between these two findings possibly arises from the phosphorylation-dependent subcellular localization of HYL1. They demonstrated that HYL1 is a nuclear-cytoplasmic shuttling protein that moves back to the cytoplasm in darkness, after performing its role in miRNA biogenesis in the nucleus during the day. Even in darkness, phosphorylated HYL1 remains in the nucleus, where it escapes the cytoplasmic protease, and thus becomes more stable, whereas dephosphorylated HYL1 moves to the cytoplasm and is degraded. Upon restoration of light, the rapid dephosphorylation of the nuclear-retained HYL1 by CPL1 leads to the reactivation of miRNA biogenesis. Furthermore, this study showed that non-phosphorylated HYL1 is rapidly cleaved by the cell-free cytoplasmic extract.

A paralog of HYL1, Double-Stranded RNA Binding Protein 4 (DRB4), functions in the plant defense system against viral infections (Jakubiec *et al.*, 2012; Lim *et al.*, 2018). DRB4 directly interacts with APC10/DCO1, a subunit of the anaphase-promoting complex (APC/C). The level of DRB4 dramatically increased in response to MG132 treatment and APC10 RNAi knock-down, suggesting that DRB4 might be a target of the APC complex-mediated UPS pathway (Marrocco *et al.*, 2012). Moreover, a recent study showed that levels of DRB4 are also positively regulated by COP1. COP1 regulated the level of both HYL1 and DRB4, which in turn regulated the level of the resistance protein Hypersensitive response to Turnip crinkle virus (HRT) that provides plants with resistance against turnip crinkle virus (TCV). Therefore,

HRT levels and, consequently, pathogen resistance, were notably reduced in a *cop1* mutant background. This study suggested that the balance between COP1 and the proteolytic regulation of HYL1 and DRB4 by an unknown protease and APC10-mediated UPS, respectively, determines the regulation of HRT in plants (Lim *et al.*, 2018); however, it has been reported that DRB4 is recruited to the cytoplasm upon TYMV infection (Jakubiec *et al.*, 2012), whereas APC10/DCO1 is known to locate in specific nuclear bodies (Eloy *et al.*, 2011). Therefore, the scenario for DBR4 protection by COP1, whether in the cytoplasm or the nucleus, should be further defined (Figure 3g,f). These studies have shown that the proteolytic regulation of components of the small RNA-mediated gene silencing pathway could be diverse and essential for light-responsiveness and the defense of plants.

CONCLUDING REMARKS

The miRNA gene-silencing pathway is one of the most versatile regulatory mechanisms in eukaryotic organisms. Current knowledge in the pathway shows that the regulation of the miRNA repertoire in a cell goes way beyond the canonical transcriptional regulation of the miRNA encoding genes. Tissue-specific sets of miRNA biogenesis cofactors, the post-translational regulation of this process, alternative processing efficiency, dependent upon pre-miRNA structure, and even the differential stability of the mature miRNA dictate, in an environment-dependent way, the collection of miRNAs that are available in a given plant tissue. In the future it will be necessary to study the pathway in a tissue-specific way or even at single-cell level to understand where and when each of the known miRNA factors act. Understanding how, where, when and what participates in the processing of each individual miRNA would be the next frontier in this research field, allowing us to create an atlas of miRNA-silencing niches in a plant.

Box 2 Open questions

- What are the subcellular localization and dynamics of each step in the miRNA pathway?
- Where are the expression niches of each miRNA cofactor and how do these affect their interactions?
- Which of the proteolytic pathways – the ubiquitin–proteasome system (UPS), autophagy or independent proteases – is involved in the destabilization of miRNA-biogenetic components?
- Are particular miRNA precursors recognized by specific regulatory proteins?

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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