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HYL1's multiverse: A journey through miRNA biogenesis and beyond canonical and non-canonical functions of HYL1

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Abstract

A delicate balance in gene expression, a process highly controlled by post-transcriptional gene silencing mediated by miRNAs, is vital during plant growth and responses to stress. Within the miRNA biogenesis pathway, HYL1 is one of the most important proteins, initially recognized for its role as a cofactor of DCL1. Yet, HYL1's functions extend beyond miRNA processing, encompassing transcriptional regulation and protein translation between other recently discovered functions. This review comprehensively examines our current knowledge of HYL1 functions in plants, looking at its structure, the complex biochemistry behind it, and its involvement in a variety of cellular processes. We also explored the most compelling open questions regarding HYL1 biology and the further perspectives in its study. Unraveling HYL1 functional details could better understand how plants grow, face environmental stresses, and how the miRNA pathway adapts its outcome to the plant growing conditions.

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Introduction

MicroRNAs (miRNAs) constitute a class of small non-coding RNAs that play pivotal roles in regulating gene expression throughout plant development and in response to various stresses. These molecules, approximately 21 nucleotides (nt) in length, originate from long RNA precursors known as pri-miRNAs, which are transcribed by RNA polymerase II (Pol II) from '*MIRNA*' genes [1,2]. What makes pri-miRNAs unique long-noncoding RNAs is their capacity to adopt a self-complementary structure resembling a hairpin recognized by the miRNA processing complex. Recognition of specific features within this structure initiates processing, primarily by DICER-LIKE1 (DCL1), which undergoes successive cleavage steps to produce a mature miRNA/miRNA* duplex. The mature miRNA guides an Argonaute (AGO) protein, causing the sequence-specific silencing of target mRNAs. In plants, this interaction often leads to AGO-mediated cleavage of the target mRNA, although translational inhibition is also observed, and in some cases, miRNAs can even trigger DNA methylation [1,3]. The mechanism of RNA-dependent gene silencing mediated by miRNAs is conserved across all eukaryotic lineages.

Although DCL1 is the main enzyme responsible for processing miRNA precursors, a plethora of co-factors have been identified to assist this process either in a tissue/condition-specific manner or more constitutively [1,2]. Among the best-characterized cofactors of DCL1, we find SERRATE (SE) and HYPONASTIC LEAVES 1 (HYL1), also known as Double-stranded RNA-Binding protein 1 (DRB1), which have long been associated with miRNA biogenesis. While SERRATE has been known for a long time to participate in several biological processes besides miRNA biogenesis [4], HYL1 was only proposed as a DCL1-cofactor for several decades. However, in recent years, an increasing body of evidence indicates that HYL1 presents broader activities in the miRNA pathway beyond its function in assisting DCL1 and even functions completely independent of this regulatory pathway. In this review, we discuss current and historical knowledge about the functions, regulation, structural (Box 1), and evolutionary (Box 2) features of HYL1 both

Box 1. Structural Features of HYL1

At the protein level, HYL1 possesses two double-stranded RNA-binding domains (dsRBD) toward its N-terminal region, a nuclear localization signal (NLS) in the middle [56], and an intrinsically disordered region composed of six nearly identical repetitions of 28 amino acids near its C-terminus.

The dsRBD domains, crucial for interaction with RNA, adopt an αββββα structure [51,52]. The protein exhibits similar affinity for pri-miRNAs, miRNA precursors (pre-miRNA), and miRNA duplexes, suggesting that once bound to a pri-miRNA, HYL1 remains associated with the miRNA encoding region during most of the miRNA lifecycle [52]. dsRBD1 shows a higher affinity for dsRNA than the second domain [51,52]. Within this domain, specific amino acids in helix 1, the β1-β2 loop, and the β3-α2 loop are essential for the interaction with dsRNA [51]. However, further investigation revealed that the β1−β2 loop in dsRBD1 is dispensable for HYL1 activity and has minimal influence on RNA-binding affinity [53]. Nonetheless, the presence of the second dsRBD is critical for the precise and strong interaction between the first dsRBD and dsRNA. In contrast to dsRBD1, the second dsRBD exhibits notable differences in its shape from the canonical dsRBD, and its primary function is associated with protein−protein interactions [52].

Since the divergence of mosses from seed plants, a strong interaction between HYL1 and DCL1 exists [54]. Although the interacting regions of both proteins have been identified, there are discrepancies in the reported results [54,55]. While the dsRBD2 was unequivocally identified as the docking point in HYL1, two independent reports differ on whether the first dsRBD of DCL1 or its DUF283 domain is involved in the interaction [54,55]. It is plausible that both interaction points contribute to the functional association of HYL1-DCL1 *in vivo*.

Regarding the C-terminal intrinsically disordered region, it was shown to be nonessential for HYL1 function during miRNA biogenesis [56]. However, it is indispensable for HYL1 homodimer formation [57]. The high amino acid conservation between repetitions, despite the variable number of repetitions depending on the plant species [14], and the phosphorylation potential of this region [14], suggest its functionality, although its specific role remains unknown. It is possible that the dimerization of HYL1 through the C-terminal domains or interaction with other proteins, processes potentially regulated by phosphorylation, control some of the HYL1 functions not related to miRNA processing. Unraveling the function of this domain of HYL1 poses an interesting question to answer in the near future.

Box 2. Conservation Features of HYL1

Besides HYL1, *Arabidopsis thaliana* contains five other related DOUBLE-STRANDED RNA BINDING proteins (DRB 2 to 5 and DRB7). HYL1 and DRB4 play crucial roles in assisting DCL1 and DCL4 during miRNA and trans-acting small-interfering (tasiRNA) biogenesis respectively [8,12]. DRB2, acts as a dual regulator of miRNA processing in specific tissues, such as the shoot apical meristem [58]. This regulation can be either synergistic or antagonistic to canonical miRNA biogenesis, depending on the pri-miRNA structure, although the precise mechanism underlying this regulation remains unclear [58]. DRB2 also appears to have an antagonistic role with DRB4 in producing PolIV-dependent siRNA [59]. DRB2, DRB3, and DRB5 have a high amino acid sequence identity and similar expression patterns in the shoot apical meristem; however, DRB3 and DRB5, unlike DRB2, are located in the cytoplasm instead of the nucleus [58]. Despite this, DRB2, DRB3, and DRB5 have a strong genetic interaction and seem to function in the same non-canonical miRNA pathway [60]. DRB7 was recently identified as a new member of this protein family in *Arabidopsis thaliana*. It is involved in the easiRNA pathway, negatively impacting the accumulation of DCL3-dependent siRNAs [61]. DRB7 competes with DCL4 for DRB4 binding to form a complex that specifically sequesters long dsRNA precursors to repress siRNA production by preventing their access and processing by the siRNA machinery [62]. Understanding the specifics of this non-canonical pathway is crucial for unraveling the functional significance of each DRB protein, its potential crosstalk with well-established pathways, and its impact on gene expression and plant development.

Unlike plants, miRNA biogenesis in animals occurs in the nucleus and cytoplasm [63]. Within the nucleus, the RNase type III Drosha processes pri-miRNAs into pre-miRNA by cleaving at the base of pri-miRNA, a process assisted by Pasha (DGCR8 in vertebrates) [64]. The processed pre-miRNA is subsequently exported into the cytoplasm and further processed by Dicer in association with other double-stranded RNA binding proteins such as loquacious (Loqs), transactivation response element RNA-binding protein (TRBP), and protein activator of the interferon-induced protein kinase (PACT) [64]. The differences in miRNA biogenesis between animals and plants and the absence of HYL1 homologs in bilaterian animals have led to the historical belief that HYL1 evolved independently in these two groups. However, homologs for HYL1 and SE were recently reported in the sponge Amphimedon and *Nematostella vectensis* [65,66], suggesting the presence of an HYL1-like protein in the last common ancestor of plants and animals. These findings challenge the notion of convergent evolution. These discoveries introduce an intriguing evolutionary connection between plants and cnidarians, adding a layer of complexity to our comprehension of microRNA pathways. It proposes the potential for a common evolutionary origin between plants and animals, reshaping our understanding of these molecular processes.

within and outside the miRNA pathway. We also explore gaps in our current understanding of this multifaceted protein and the most compelling questions and future perspectives in its study.

HYL1 functions in miRNA biogenesis: beyond pri-miRNA processing

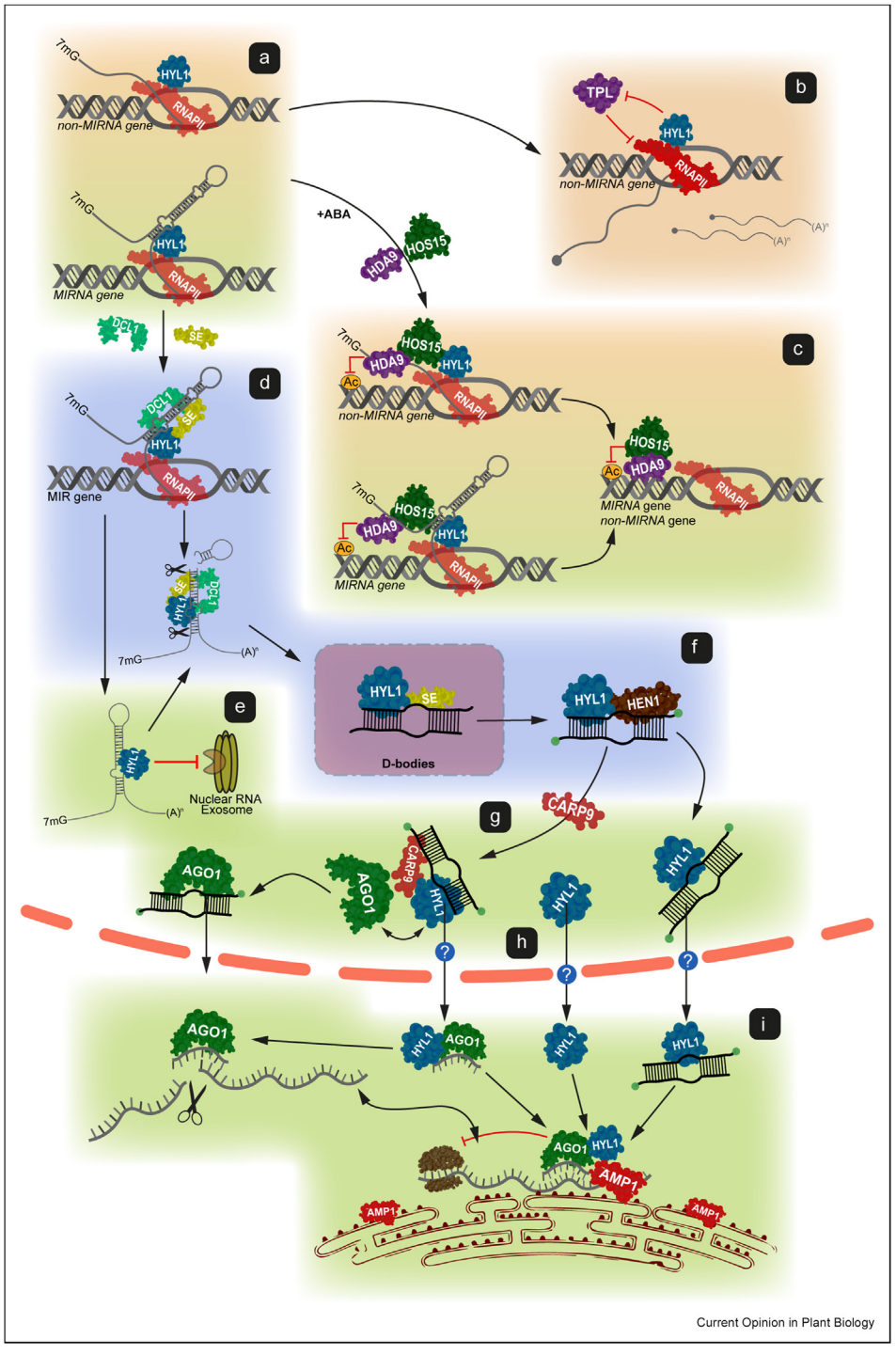
HYL1 was discovered over 20 years ago when it was linked to the response of *Arabidopsis thaliana* plants to

abscisic acid, auxin, and cytokinin [5]. Those first reports showed that *hyl1* mutants exhibited a pleiotropic phenotype characterized by shorter stature, leaf hyponasty, delayed flowering, reduced fertility, impaired apical shoot development, and altered root growth [5]. Shortly afterward, HYL1 was associated with miRNA production [6,7] by interacting with DCL1 [8] within D-bodies [9,10]. Consistently, *hyl1* mutants exhibit reduced levels of mature miRNAs, associated with elevated levels of unprocessed pri-miRNAs and deregulation of miRNA-targeted mRNAs [6,7,12]. Early biochemical research on HYL1 revealed that its interaction with DCL1 promotes miRNA processing accuracy [11,12] and strand sorting [13,14]. However, later studies showed that DCL1-mediated miRNA processing could efficiently occur independently of HYL1, at least under low-temperature conditions, while some pri-miRNAs appear to be naturally independent of this protein for still unclear reasons [15,16]. The temperature effects over the folding structure of the pri-miRNAs likely dictate the requirement of HYL1 during miRNA processing. Furthermore, a genetic screen identified a point mutation in the DCL1 encoding gene, rendering the protein independent from HYL1 activity during miRNA processing [17]. These results suggest that HYL1 may act by controlling DCL1 structure, allowing proper pri-miRNA processing. Alternatively, this point mutation in the DCL1 could potentially lead to improved stabilization of pri-miRNAs upon binding. As a result, DCL1 becomes less reliant on HYL1 during miRNA processing. Still, new evidence suggests that HYL1 not only acts in the miRNA pathway by modulating DCL1 activity but also at other levels. For example, a recent study found that HYL1 is associated with Pol II independently of DCL1 and SE, controlling the transcription initiation and elongation of *MIRNA* genes [18] (Figure 1a). This discovery implies that HYL1 may already be associated with miRNA loci during pri-miRNA transcription, helping the processing complex assemble around nascent pri-miRNAs as soon as the stem-loop structure forms, explaining the efficient co-transcriptional processing of many miRNAs [19] (Figure 1d). In agreement, HYL1 was found to associate with *MIRNA* loci by recognizing and binding nascent pri-miRNAs [20]. This association, along with the interaction with the HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 15 (HOS15)/HISTONE DEACETYLASE9 (HDA9) complex, promotes deacetylation and repression of specific *MIRNA* loci, providing feedback regulation of miRNA expression under ABA treatment [20] (Figure 1c). Furthermore, HYL1 association with pri-miRNAs, relying on protein homo-dimerization [21], also protects them from nuclear exosome-mediated decay [22] (Figure 1e). However, in the absence of the homolog of the yeast A1-alpha2 repressing protein (AAR2), HYL1 may also cause the degradation of pri-miRNAs through an unknown mechanism [23]. Although apparently contradictory, these reports show that HYL1's function within the miRNA processing complex extends beyond controlling DCL1 processing accuracy and directly affects the homeostasis and turnover of pri-miRNAs.

HYL1 is also a key player in the processing of non-chromatin-associated pri-miRNAs, also refer as post-transcriptional processing. Such processing likely occurs within nuclear speckles known as D-bodies where HYL1 was shown to participate in miRNA processing and to mobilize, associated with the mature miRNA duplexes, outside these nuclear speckles once processing is completed [24] (Figure 1d). Several proteins, including DEAD-box helicases RCF1, RH6, RH8, and RH12, the THO/TREX complex, enhance HYL1's affinity for pri-miRNAs, promoting D-bodies formation and miRNA processing [25–28]. Interestingly FORKHEAD-ASSOCIATED DOMAIN 2 (FHA2) also boosts HYL1's affinity for pri-miRNAs, but in this case miRNA processing is impaired as the consequence of a paralleled reduction in DCL1 affinity for pri-miRNAs [27].

After pri-miRNA processing, miRNA/miRNA* duplexes are released from the D-bodies; they remain bound to HYL1 [24,29]. It remains unclear whether this holds true for both co- and post-transcriptionally processed miRNAs. Possibly before exiting the D-bodies, SE releases the miRNA duplex, facilitating the interaction of the miRNA/miRNA* duplex with HUA ENHANCER1 (HEN1) to catalyze the 2′-O-methylation of the miRNA duplex's 3′ ends [29,30] (Figure 1f). The dynamics of these steps regarding where they happen are still unclear. In a final nuclear step, CONSTITUTIVE ALTERATIONS IN THE SMALL RNAs PATHWAYS9 (CARP9), through interaction with HYL1, mediate the transfer of the miRNA duplex to AGO1, facilitating nuclear export of miRNA [31,32] (Figure 1g). As AGO1-loaded miRNAs appear to act only cell-autonomously [33–35], it is unclear which and how miRNAs are selected to be transferred to AGO1 and which remain and move to the cytoplasm unloaded. A large pool of AGO1-unloaded miRNAs exists in the cytoplasm [36] and is thought to be responsible for the non-cell-autonomous functions of miRNAs. Whether these miRNAs exit the nucleus still associated with HYL1, which localizes in both the nucleus and cytoplasm, and whether HYL1 has any role during miRNA systemic movement is unknown and worth studying in the future (Figure 1h). Cytoplasmic HYL1, interacting with ALTERED MERISTEM PROGRAM1 (AMP1) and AGO1 in the endoplasmic reticulum, regulates miRNA-mediated inhibition of mRNA translation by facilitating the distribution of AGO1 in polysomes [37] (Figure 1i). One open question here is whether this function is achieved by assisting miRNA-loaded AGO1 or directly through HYL1 association with miRNAs. If this is the case, the nuclear association of HYL1 with the mature

Figure 1



HYL1 functions throughout the cell. HYL1 can associate with Pol II to regulate *MIRNA* and *non-MIRNA* gene transcription **(a)**. Through this interaction, HYL1 impairs TPL repressor complex activity, thereby promoting the transcription of non-miRNA genes **(b)**. Under ABA treatment, HYL1 interaction with Pol II acts as a scaffold recruiting the HDA9/HOS15 complex to the associated loci, triggering histone deacetylation and the repression of gene expression **(c)**. In turn, the binding of HYL1 to nascent pri-miRNAs promotes the assembly of the processing complex and facilitates the co-transcriptional processing of miRNAs by interacting with DCL1 and SE, increasing the processing accuracy **(d)**. Additionally, HYL1 binding with pri-miRNAs protects them from nuclear RNA exosome-mediated degradation **(e)**. Within D-bodies, miRNA/miRNA* duplexes remain associated with HYL1 and SE after processing, leaving these speckles still bound to HYL1, further allowing their interaction with HEN1 to undergo the 2′-O-methylation of the 3′ end of the miRNA duplex **(f)**. Mature miRNAs associated with HYL1 interact then with CARP9, facilitating the transfer of the miRNA duplexed to AGO1, which retains the guide strain and exits the nucleus already loaded with the miRNA where it targets mRNA for their silencing **(g)**. HYL1 shuttles to the cytoplasm. Whether free, associated with a mature miRNA duplex, or interacting with AGO1 is unclear **(h)**. In any case, cytoplasmic HYL1 can interact with AGO1

miRNA duplex gains relevance in the cytoplasm. Then, it would also be important to study whether this fraction of cytoplasmic HYL1-associated miRNAs remains double-stranded or a strand is discarded, paralleling RISC maturation.

The reports discussed in this section demonstrate that HYL1 acts in the miRNA pathway from as early as *MIRNA* transcription to as late as cytoplasmic translation repression of miRNA targets, making this protein one of the most important players in this pathway and a potential regulatory hub for miRNA-mediated gene silencing.

Homeostasis and turnover of HYL1

The homeostasis, activity, and turnover of HYL1 are greatly controlled by its subcellular distribution, which appears to depend on its phosphorylation, although other post-translational modifications still to be identified likely have similar regulatory effects. While the nuclear import of HYL1 from the cytoplasm was attributed to the importin KARYOPHERIN ENABLING THE TRANSPORT OF CYTOPLASMIC HYL1 (KETCH1) [38], how it is exported to the cytoplasm is unknown, although it may involve the EXPO1 complex, which participates in AGO1 nuclear export [31], in the hypothetical case that HYL1 exit the nucleus associated with AGO1 (Figure 2). The functions of HYL1 in the nucleus were largely studied, including most of the activities described in the previous section. Conversely, the roles of HYL1 in the cytoplasm are largely unknown besides the previously mentioned regulation of miRNA-mediated translation inhibition in the ER. Nevertheless, cytoplasmic HYL1 undergoes proteolytic degradation, particularly during darkness [39,40]. During this process, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), which is located in the cytoplasm during the day, protects HYL1 from degradation by interacting with the protease HYL1-CLEAVAGE SUBTILASE 1 (HCS1), preventing its interaction with HYL1 [41]. During the night or extended periods of insufficient light, COP1 moves into the nucleus, leaving HCS1 free to interact with the cytoplasmic fraction of HYL1 and trigger its degradation [40,41]. Additionally, HYL1 degradation in the cytoplasm is promoted by AAR2 but this process seems to be independent of light or dark conditions [23]. Buffering the day/night miRNA regulation, FHA2, which is unstable during the night, inhibits miRNA biogenesis by interacting with DCL1, preventing its association with pri-miRNAs. However, FHA2 also interacts with HYL1, enhancing its affinity for pri-miRNAs. This opposite effect over the pri-miRNA affinity of DCL1 and HYL1 controlled by FHA2 is intriguing [27] (Figure 2).

Interestingly, although miRNA biogenesis is impaired at night due to active degradation of HYL1, a large pool of this protein remains sequestered in the nucleus in an inactive state [39]. Such nuclear isolation prevents HCS1-mediated degradation of HYL1, forming a reserve pool of inactive protein that can be quickly reactivated when light becomes available [39]. Phosphorylation of HYL1, which prevents its nuclear export but renders the protein inactive, at least in its miRNA processing functions, contributes to this protection [14,39].

Until now, phosphorylation is the only characterized post-translational modification of HYL1. It affects its activity during miRNA processing, stability, subcellular localization, capacity to interact with other proteins and RNA, and formation of D-bodies. Several proteins and phosphatases, including PROTEIN PHOSPHATASE 4 (PP4), SUPPRESSOR OF MEK 1 (SMEK1), AAR2, REGULATOR OF CBF GENE EXPRESSION 3 (RCF3), and C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 (CPL1), promote HYL1 dephosphorylation, activating the proteins [14,23,42,43]. Conversely, FYVE DOMAIN PROTEIN REQUIRED FOR ENDOSOMAL SORTING 1 (FREE1), which negatively affects CPL1 activity, MAP PROTEIN KINASE 3 (MPK3), which phosphorylates HYL1's Ser42 in a non-canonical site, and SNF1-RELATED PROTEIN KINASE 2 (SnRK2) trigger HYL1 phosphorylation and its inactivation/degradation [[44–47] (Figure 2)].

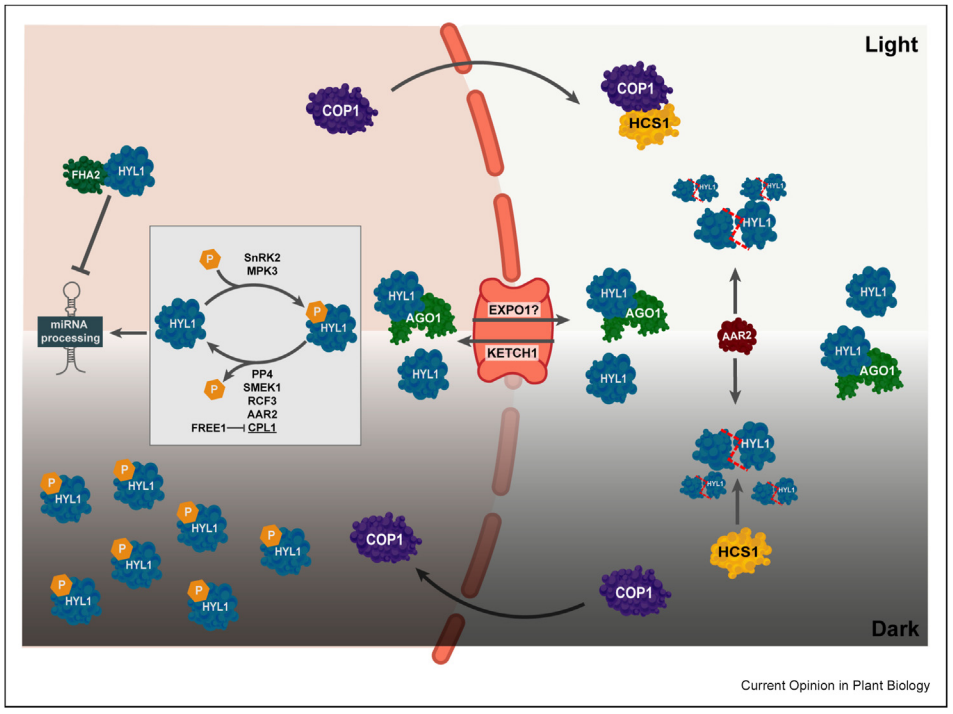
HYL1, like many miRNA-biogenesis-related proteins, responds to ABA-signaling, and its mutants are insensitive to this phytohormone [5]. Notably, several proteins that control HYL1 homeostasis also respond to ABA signaling, suggesting that HYL1 can be a regulatory hub for ABA-mediated miRNA regulation. Such regulation may play a critical role during ABA-mediated developmental adaptations and physiological processes controlled by ABA, such as seed germination, a process altered in HYL1 mutants [5].

It is not all about miRNAs: non-canonical functions of HYL1

Except for early reports describing the discovery of HYL1, all studies about this protein during the last 20 years explored HYL1 within the orbit of its functions in the miRNA pathway. Recently, new evidence has emerged pointing to unexpected functions of this protein in processes unrelated to its canonical actions in the miRNA pathway. For example, HYL1 activity, but not DCL1 or SE, was associated with developmental reprogramming during skotomorphogenesis [48,49]. It was found that a pool of phosphorylated HYL1 affects

and AMP1 to promote miRNA-mediated translational inhibition by altering the distribution of AGO1 in polysomes **(i)**. Blue areas of the image denote HYL1 activities strictly related to DCL1-mediated miRNA processing; green areas represent functions related to the miRNA pathway but not necessarily miRNA biogenesis; while orange areas indicate potential functions outside the miRNA pathway.

Figure 2



Keeping the Balance: regulation of HYL1 turnover. In the nucleus, HYL1 undergoes phosphorylation by kinases SnRK2 and MPK3, rendering it inactive in miRNA processing. A consortium of proteins, including PP4, SMEK1, RCF3, AAR2, and CLP1, collaborates to ensure sustained levels of active, dephosphorylated HYL1. Among these, CPL1 activity is negatively modulated by FREE1. FHA2, stable in light conditions, binds to HYL1, reducing its affinity for dsRNA and diminishing pri-miRNAs processing efficiency. The shuttling of HYL1 between the nucleus and cytoplasm is orchestrated by KETCH1, facilitating HYL1 import into the nucleus and likely EXPO1 complex in HYL1 export, potentially in conjunction with AGO1. In the cytoplasm, during daylight, COP1 interacts with HCS1, a protease, inhibiting HYL1 degradation. Conversely, in the absence of light, COP1 translocates to the nucleus, triggering HCS1-mediated HYL1 degradation. AAR2 also contributes to HYL1 degradation but in a light-independent manner. In addition to its role in inhibiting miRNA processing, HYL1 phosphorylation hinders its cytoplasmic export, leading to the formation of a nuclear pool of inactive protein protected from dark-induced degradation.

the stability of ELONGATED HYPOCOTYL 5 (HY5), a key regulator in photomorphogenic growth [48]. Complementation experiments using a truncated version of HYL1 containing only the first double-stranded RNA binding domain (dsRBD) showed that mutant plants expressing this version of HYL1 do not present miRNA biogenesis defects but still show aberrant development during skotomorphogenesis, suggesting that the second dsRBD domain is required for such HYL1 function [48]. Similarly, in *hyl1* mutants, the auxin gradient controlling apical hook elongation is affected in a miRNA-independent manner [49].

HYL1 controls miRNA abundance at the transcriptional level by controlling *MIRNA* encoding genes expression [18,20]. Notably, this function extends beyond *MIRNA* genes by regulating other coding genes. HYL1 interaction with Pol II and certain transcription factors of the TOPLESS family results in altered expression of many non-miRNA genes in *hyl1* mutants, an alteration not paralleled in *dcl1* or *se* mutants, showing its independence from the miRNA pathway [18] (Figure 1b). Following the role of HYL1 in skotomorphogenesis, a gene ontology analysis showed that genes regulated by HYL1 are commonly related to light signaling and chloroplasts [18]. Although these regulatory effects are likely caused by the interaction of HYL1 with transcription factors or nascent RNA molecules, it was reported that the C-terminal intrinsically disordered region of HYL1 has DNA-binding capacity, potentially allowing it to act as a transcription factor directly [50]. However, this last HYL1 capacity needs further confirmation using an *in vivo* experimental setup.

Conclusions and open questions

During the past decade, HYL1 progressively left its status as a dedicated DCL1 cofactor to become a central component of the entire miRNA regulatory pathway, prompting numerous research endeavors to explore its potential as a regulatory hub for miRNA activity. However, many questions remain unanswered. For instance, the precise mechanisms underlying HYL1's involvement in the various processes it influences remain elusive. Even in its well-studied role as a DCL1 cofactor,

the exact biochemical mode of action of HYL1 remains largely unknown. Furthermore, in the future, it will be crucial to discover which cellular receptors and signal transduction pathways control HYL1's phosphorylation, especially during stressful conditions. The association of HYL1 with miRNA duplexes opens the possibility that this protein contributes to the subcellular and non-cell-autonomous distribution of miRNAs. However, this is a completely unexplored hypothetical scenario that requires further investigation. Exploring the biochemical and molecular consequences of HYL1 interactions with other proteins, such as COP1, CARP9, AGO1, or AAR2, may shed light on its functional mechanisms and stability. The structural features of HYL1, particularly its intriguing and functionally elusive C-terminal domain, hold promise for understanding HYL1 conservation and its non-canonical functions. By deciphering these puzzle pieces, we can gain insights into HYL1's role in plant growth and stress response and potentially unravel clues to explain the divergence in miRNA pathways between plants and metazoans.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Pablo Manavella reports financial support was provided by Spanish Ministry of Science and Innovation. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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