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MicroRNAs in the moss *Physcomitrella patens*

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Abstract Having diverged from the lineage that lead to flowering plants shortly after plants have established on land, mosses, which share fundamental processes with flowering plants but underwent little morphological changes by comparison with the fossil records, can be considered as an evolutionary informative place. Hence, they are especially useful for the study of developmental evolution and adaption to life on land. The transition to land exposed early plants to harsh physical conditions that resulted in key physiological and developmental changes. MicroRNAs (miRNAs) are an important class of small RNAs (sRNAs) that act as master regulators of development and stress in flowering plants. In recent years several groups have been engaged in the cloning of sRNAs from the model moss *Physcomitrella patens*. These studies have revealed a wealth of miRNAs, including novel and conserved ones, creating a unique opportunity to broaden our understanding of miRNA functions in land plants and their contribution to the latter's evolution. Here we review the current knowledge of moss miRNAs and suggest approaches for their functional analysis in *P. patens*.

Keywords AGO1 · DCL1 · Development · Evolution · Gametophyte · MicroRNA · Moss · *Physcomitrella patens* · Stress

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Introduction

MicroRNAs, are genome-encoded noncoding RNAs of ~21 nucleotides (nt) in length that act as repressors of target genes in animals and plants (Bartel 2004). A mature miRNA and its passenger strand (miRNA*) are derived from opposing arms of a hairpin precursor (pre-miRNA) found in a longer primary transcript (pri-miRNA) that is transcribed from *MIR* genes by RNA polymerase II (Chen 2008). In *Arabidopsis thaliana*, release of a pre-miRNA from the pri-miRNA and its subsequent processing into a mature miRNA/miRNA* duplex occurs via at least two cleavage steps, which are catalyzed by the RNaseIII-type enzyme DICER-LIKE 1 (DCL1) (Kurihara et al. 2006; Kurihara and Watanabe 2004; Park et al. 2002) assisted by HYPONASTIC LEAVES1 and SERRATE (Dong et al. 2008; Kurihara et al. 2006). Following its release, the miRNA/miRNA* duplex is methylated by HUA ENHANCER 1 (Yu et al. 2005). Then, its two strands are separated and the single-stranded mature miRNA is specifically sorted into an ARGONAUTE (AGO) protein that form the core of an effector complex called RNA-induced silencing complex (RISC) (Mi et al. 2008). AGO1 is considered the major miRNA-RISC. It performs slicer activity that cleaves miRNA targets (Baumberger and Baulcombe 2005; Qi et al. 2005) and preferentially associates with 21-nt-long sRNAs that have a 5′ terminal uridine (Mi et al. 2008; Montgomery et al. 2008), features which are characteristic of most plant miRNAs. Loaded miRNAs guide RISC to nearly complementary target sequences resulting in their cleavage (Llave et al. 2002; Tang et al. 2003) or translation repression (Brodersen et al. 2008; Chen 2004). Accumulating evidence on miRNA functions in *A. thaliana* and other model flowering plants suggests that they play critical roles in vegetative and reproductive development (Chen 2009), nutrient homeostasis, response to

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environmental stresses (Sunkar 2010), and autoregulation of the miRNA pathway itself (Vaucheret et al. 2006; Xie et al. 2003).

Bryophytes are believed to have shared a common ancestor with flowering plants ~400 million years ago (MYA) (Kenrick and Crane 1997). *Physcomitrella patens* (Bryopsida) is a monoecious moss that has emerged as a useful model plant mainly because it is easily transformed and performs efficient homologous recombination, which allows the study of gene function by targeting gene disruptions or replacements (Cove 2005; Schaefer 2001). Moreover, its complete genome sequence (~511 Mb) was recently published (Rensing et al. 2008) and powerful molecular tools are available (Frank et al. 2005a). Unlike vascular plants (ferns and seed plants), the life cycle of *P. patens* like all mosses is dominated by a haploid gametophyte phase (reviewed in Reski 1998) making the analysis of engineered loss-of-function *P. patens* mutants straightforward. Furthermore, the *P. patens* gametophyte has simple tissue morphology with only a few cell types, which facilitate the characterization of abnormal mutant phenotypes and the study of plant development (Prigge and Bezanilla 2010).

The development of the dominant *P. patens* gametophyte, which is larger and more complex than the sporophyte, can be divided into two distinct stages: the protonema (juvenile gametophyte) and the gametophore (adult gametophyte) (Fig. 1). The protonema, generated by spore germination, is composed of filaments of cells that extend by successive divisions of their tip cell (Menand et al. 2007). Young protonema filaments have assimilatory functions and consist of chloronema cells that are densely packed with large chloroplasts and have perpendicular cell walls (Fig. 1a). These filaments extend until, in response to increases in light (Cove and Ashton 1988) and auxin (Johri and Desai 1973) their tip cells differentiate into caulonema cells, which are longer, divide more often, contain fewer smaller chloroplasts and have oblique cell walls (Fig. 1b). Soon after the division of a caulonema tip cell, an initial cell is formed in the second subapical cell. In the presence of cytokinin this initial cell, instead of producing a lateral filament, will divide and produce a bud, marking the transition from juvenile to adult gametophyte stage (Schumaker and Dietrich 1998) (Fig. 1c). By meristematic growth, this bud forms a leafy gametophore on which female and male gametangia will later develop (Fig. 1d). Once fertilized, the zygote will develop into a tiny diploid sporophyte that produces haploid spores (Fig. 1e).

Cloning efforts have identified numerous miRNAs from various *P. patens* gametophyte tissues, many of which target regulatory genes. Furthermore, certain miRNA families and their corresponding targets have been found to be conserved with flowering plants, suggesting a common origin of miRNA-regulated pathways in land plants (Axtell and Bowman 2008). In this article, we review what is known about *P. patens* miRNAs and discuss how miRNA functions and regulatory roles may be elucidated in this unique model plant.

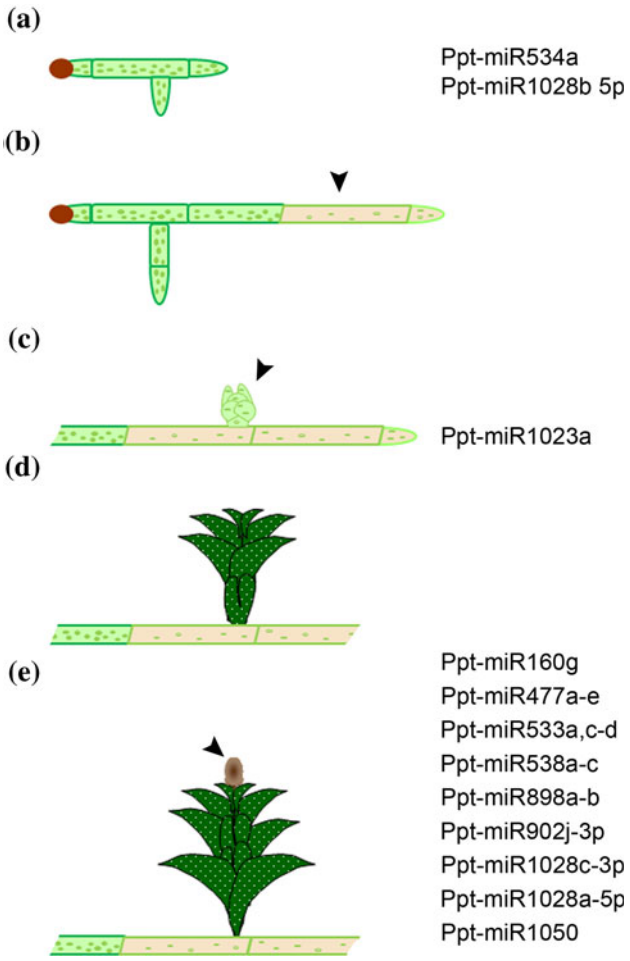

Fig. 1 A cartoon of major stages in *P. patens* gametophyte development. The juvenile gametophyte stage is initiated by the germination of a haploid spore (*circle*) to form a chloronema filament (**a**). Under certain conditions, a tip chloronema cell will differentiate into a caulonema cell (*marked by an arrowhead*, **b**). **c** In the presence of cytokinin a juvenile gametophyte undergoes transition indicated by the formation of a bud (*marked by an arrowhead*). This bud later develops into an adult gametophyte or gametophore that bears gametangia (**d**). Upon egg fertilization a diploid sporophyte is formed (**e**, *marked by an arrowhead*). Relatively abundant miRNAs, which are differentially expressed between the stages **a**, **c** and **e** (Table 1) are indicated next to the stage with the highest expression (Axtell et al. 2007)

miRNA discovery in *P. patens*

The first experimental evidence of miRNA-guided target cleavage in a non-flowering plant was provided by Floyd

and Bowman (2004). They cloned the class III HD-ZIP gene homolog *PpC3HDZIP1* from *P. patens*, and found that it contains a conserved miR166-target sequence. Using 5′ rapid amplification of complementary DNA ends (5′-RACE) they were able to clone a putative miR166-guided cleavage product of *PpC3HDZIP1* mRNA (Floyd and Bowman 2004). This suggested that miR166 negatively regulates *PpC3HDZIP1* expression in *P. patens* and provided indirect evidence for the presence of miR166 in this basal land plant.

Soon after, the cloning of miR160 and miR160-guided cleavage products of auxin response factor (*ARF*) genes from the leafy gametophyte of the moss *Polytrichum juniperinum* was reported (Axtell and Bartel 2005). This provided direct evidence that miR160 is present and functional in moss. In *A. thaliana*, miR160 regulates *ARF10*, *ARF16* and *ARF17* (Liu et al. 2007; Mallory et al. 2005; Wang et al. 2005) indicating that some miRNA: target interactions were deeply conserved during land plant evolution.

Three groups used a conventional sRNA cloning approach to identify miRNAs from *P. patens* (Arazi et al. 2005; Fattash et al. 2007; Talmor-Neiman et al. 2006a). Arazi et al. (2005) identified, among 100 cloned protonema (Fig. 1a–c) sRNAs, homologs of miR390, miR156, miR319 and miR535, revealing their ancient origins within the land plant lineage. In addition, that study reported the cloning of five *P. patens*-specific miRNAs based on northern hybridization and the identification of corresponding pre-miRNAs among expressed sequence tags (ESTs) (Arazi et al. 2005). The release of *P. patens* whole-genome shotgun (WGS) sequences led to the identification of an additional 31 miRNA families, based on predicted hairpin structure surrounding cloned sRNAs or their homology to known miRNAs, from the same library (Talmor-Neiman et al. 2006a) and from a mixed protonema (Fig. 1a)-gametophore (Fig. 1c–d) sRNA library (Fattash et al. 2007). These studies increased the number of identified miRNA families in *P. patens* to 40, nine of which were deeply conserved with flowering plants.

The introduction of next-generation sequencing technology and the parallel completion of the *P. patens* draft genome sequence (Rensing et al. 2008) paved the way for a more comprehensive exploration of its miRNA repertoire. Deep sequencing of *P. patens* sRNAs from three major developmental stages (Fig. 1a, c, e) yielded 127,135 unique sRNAs that matched the *P. patens* genome (Axtell et al. 2006). Computational analysis to predict pre-miRNA-like hairpin structures with suitable miRNA and miRNA* strands resulted in the confident identification of 97 miRNA families (Axtell et al. 2007). These included three additional deeply conserved miRNAs and all except 11 of the previously identified miRNAs by Fattash et al. 2007. Two of those 11 miRNAs (Ppt-miR414 and Ppt-miR419) are currently under review and one (Ppt-miR167) has not yet been experimentally validated. Together, the above cloning efforts identified 233 *P. patens* miRNAs corresponding to 106 unique miRNA families. Of these, 13 families (miR156, miR160, miR166, miR167, miR171, miR319, miR390, miR395, miR408, miR477, miR529, miR535, miR894) are conserved with one or more angiosperms. Bryophytes are thought to have shared a common ancestor with flowering plants ~400 MYA (Kenrick and Crane 1997) suggesting that these miRNAs have played regulatory roles since that period (Axtell and Bowman 2008). One family (Ppt-miR536) is conserved with lycopods, and the remaining 93 families are considered moss-specific, since they have not yet been cloned from any other land plant. This number of miRNA families is comparable to the 131 miRNA families identified in *A. thaliana* (miRBase release version 16), indicating that the degree of miRNA regulation in moss, a basal plant, is at least equal to that in a flowering plant.

As in flowering plants, *P. patens* miRNAs are encoded by either single (75/106) or multiple (31/106) genomic loci and their predicted pre-miRNAs vary is size and shape (Axtell et al. 2007; Fattash et al. 2007; Talmor-Neiman et al. 2006a). In addition, around 21% of them are arranged in closely linked clusters of two to three foldbacks that usually code for members of the same miRNA family (Axtell et al. 2007). This is comparable to the ratio found in a recent study of *MIR* gene clustering in *A. thaliana*, *Populus* and *Oryza* (Merchan et al. 2009). However, in contrast to angiosperm *MIR* genes that are usually found in intergenic regions, around half of the *P. patens* pre-miRNAs overlap with the positions of protein-encoding loci as annotated in the draft genome assembly (Axtell et al. 2007). Axtell et al. (2007) noted that this arrangement is reminiscent of *MIR* genes of the unicellular alga *Chlamydomonas reinhardtii*, many of which are found in introns of protein-encoding loci (Molnar et al. 2007; Zhao et al. 2007).

Most cloned *P. patens* miRNAs (79%) are 21 nt long and start with a uracil (60%), similar to higher plant miRNAs. Nevertheless, a significant fraction of them (24%) start with a cytosine, compared to only 9% of *A. thaliana* miRNAs. This suggests a major role in the miRNA pathway for a *P. patens* AGO that favors 5′ terminal cytosine and might represent a functional homolog of AtAGO5, previously shown to have a binding preference for sRNAs that initiate with cytosine (Mi et al. 2008).

miRNA biogenesis in *P. patens*

A. thaliana encodes four DICER-like (DCL) proteins (AtDCL1-4) that function with partial redundancy in

different sRNA pathways (Bouche et al. 2006; Deleris et al. 2006; Gasciolli et al. 2005). AtDCL1 is the predominant DICER enzyme processing pre-miRNAs to release miRNA/miRNA* duplexes (Kurihara and Watanabe 2004; Park et al. 2002). Consistent with its primary role in miRNA maturation, *Atdcl1* mutants are impaired in miRNA production (Kurihara et al. 2006; Kurihara and Watanabe 2004; Park et al. 2002), ectopically express miRNA target genes (Kasschau et al. 2003) and show a range of developmental phenotypes, including embryo lethality in complete loss-of-function mutants (Schauer et al. 2002). The *P. patens* genome encodes four DICER-like proteins (PpDCL), two of which (PpDCL1a and PpDCL1b) are related to AtDCL1, one to AtDCL3 and one to AtDCL4 (Axtell et al. 2007). Khraiwesh et al. (2010) generated Δ*PpDCL1a* null mutants and found that they accumulate reduced miRNA and increased target mRNA levels, indicating that PpDCL1a is required for miRNA biogenesis and is a functional ortholog of AtDCL1. Furthermore, Δ*PpDCL1a* plants displayed severe developmental phenotypes, including a protonema-stage arrest, emphasizing the importance of the miRNA pathway for normal gametophyte development (Khraiwesh et al. 2010).

The *A. thaliana* pre-miRNA hairpins are processed by AtDCL1 via at least two distinct mechanisms: a canonical loop-last mechanism (Kurihara et al. 2006; Kurihara and Watanabe 2004), and a loop-first mechanism currently found to be used for processing of the longer pre-miR159 and pre-miR319 hairpins (Addo-Quaye et al. 2009; Bologna et al. 2009). Degradome data suggest that both processing mechanisms exist in *P. patens*. Most *P. patens* hairpins are processed by the canonical loop-last mechanism (Addo-Quaye et al. 2009), whereas processing of the pre-miR319 precursors is conserved with *A. thaliana* and rice in being performed in a loop-first sequential manner, releasing miR319/miR319* and two additional miRNA/miRNA*-like duplexes (Addo-Quaye et al. 2009; Talmor-Neiman et al. 2006a). These findings suggest that PpDCL1a is biochemically similar to AtDCL1. In addition, the *A. thaliana* pre-miR319a was utilized to correctly express artificial miRNAs in *P. patens* (Khraiwesh et al. 2008), further supporting the biochemical similarities between the DCL1s of these two land plants.

It was suggested that PpDCL1b functions differently from PpDCL1a and AtDCL1 (Khraiwesh et al. 2010). Δ*PpDCL1b* mutants accumulated normal levels of miRNAs but exhibited compromised cleavage of miRNA targets, indicating that PpDCL1b is not required for miRNA biogenesis but is essential for miRNA-directed target cleavage (Khraiwesh et al. 2010). The exact function of PpDCL1b is currently unknown, but it is expected to be unique among known plant DCL1s. A proposed function is that similar to certain animal DICERs, which are components of an RNA-loading complex that loads sRNA into RISC, PpDCL1b may assist in miRNA loading into RISC (Khraiwesh et al. 2010).

Functions of specific *P. patens* miRNAs

At present the biological functions of most *P. patens* miRNAs are unknown. The available deep-sequencing data on *P. patens* miRNAs (Axtell et al. 2007) were collected separately from tissues representing three major stages in the life cycle of *P. patens*: the juvenile gametophyte stage represented by one-week old protonemata (Fig. 1a); the juvenile gametophyte undergoing transition to the adult stage, represented by two-week old protonemata with young gametophores (Fig. 1c); and the mature gametophyte and sporophyte stages, represented by 2-months-old mature gametophores bearing sporophytes (Fig. 1e). Although sample preparation for small RNA deep sequencing involves two steps of RNA adaptor ligation and one step of PCR amplification, which may introduce bias to the final numbers of certain miRNAs (Willenbrock et al. 2009), these data provide an initial estimation of the abundance and sites of activity of sequenced miRNAs in the course of gametophyte development. In addition, 5′-RACE and degradome analysis have identified 71 RNAs that serve as targets for 35 miRNA families (Addo-Quaye et al. 2009; Arazi et al. 2005; Axtell et al. 2007; Fattash et al. 2007; Talmor-Neiman et al. 2006a). These targets provide clues to the potential pathways and biological processes regulated by miRNAs in *P. patens*.

Tissue-specific abundance of *P. patens* miRNAs

It was postulated that rare miRNAs may represent recently evolved "young" miRNAs, many of which are probably functionless, since a target mRNA could not be predicted or validated for them (Fahlgren et al. 2007; Rajagopalan et al. 2006). A total of 185 miRNAs were deep-sequenced but only 79 were cloned more than 500 times, thus representing relatively abundant and most likely functional miRNAs (Axtell et al. 2007). We found that the abundance of 22 out of the 79 relatively abundant miRNAs was significantly changed (>threefold) in at least one of the three sRNA libraries, supporting differential expression during moss development (Table 1). The rest of the miRNAs showed moderate changes between the above stages or were constitutively expressed (Axtell et al. 2007). Two miRNAs (Ppt-miR534a and Ppt-miR1028b-5p) were abundant in young protonemata and almost absent from mature gametophores, suggesting that they function mainly during the juvenile gametophyte stage (Fig. 1a). The protonema-specific expression pattern of Ppt-miR534 was

Table 1 Differentially expressed *P. patens* miRNAs

miRNA name	Fold change[a]			Annotation of protein encoded by validate target mRNA[b]
	P	PG	GS	
Ppt-miR534a	4.9	1.0	0.1	BLADE ON PETIOLE 2-like BTB and ankyrin-domain proteins
Ppt-miR1028b-5p	3.8	1.0	0.1	Unknown
Ppt-miR1023a-5p	1.0	10.2	0.3	Similar to STOP1 (sensitive to proton rhizotoxicity 1)
Ppt-miR1023a-3p	1.0	7.8	0.2	
Ppt-miR160 g	0.0	1.0	4.4	Auxin response factor
Ppt-miR477a-e	0.1	1.0	5.3	Zinc-finger CCT-domain proteins
Ppt-miR533a,c,d	0.1	1.0	14.1	
Ppt-miR538a-c	1.0	0.9	3.6	MADS-box, K-box containing transcription factors
Ppt-miR898a-b	1.0	1.0	3.6	Protein kinase
Ppt-miR902j-3p	0.4	1.0	39.7	
Ppt-miR1028c-3p	1.0	0.6	38.3	Similar to protein arginine N-methyltransferase
Ppt-miR1028a-5p	1.0	0.0	16.4	Unknown
Ppt-miR1050	0.2	1.0	7.9	G-protein coupled photoreceptor/signal transducer

Calculated from the deep-sequencing data published by Axtell et al. 2007

[a] Fold change relative to median abundance. P-1 week old protonema, PG-2 weeks old protonema with young gametophores, GS-2 months old gametophores with sporophytes

[b] According to (Axtell et al. 2007) and (Addo-Quaye et al. 2009)

previously confirmed by northern hybridization (Arazi et al. 2005), supporting the authenticity of the deep-sequencing results. Ppt-miR534a guides the cleavage of two *P. patens* transcripts (Addo-Quaye et al. 2009; Axtell et al. 2007) encoding homologs of *A. thaliana* BLADE-ON-PETIOLE 1 (BOP1) and BOP2 transcriptional activators which promote cell differentiation events in the proximal regions of lateral organs (Hepworth et al. 2005; Ha et al. 2003; Jun et al. 2010; Xu et al. 2010). A single miRNA (Ppt-miR1023a) was highly abundant in 14-days-old protonemata with young gametophores (Fig. 1c), and its numbers dropped sharply (at least tenfold) in young protonemata and mature gametophores (Table 1), suggesting strong specificity to buds or very young gametophores which are unique to the transition from juvenile to adult gametophyte stage (Fig. 1c). This miRNA guides the cleavage of two *P. patens* mRNAs encoding putative zinc finger transcription factors with partial similarity to the Sensitive to Proton Rhizotoxicity1 (STOP1) zinc-finger protein, which is involved in the signal-transduction pathway of acid soil tolerance in *A. thaliana* roots (Iuchi et al. 2007). The rest of the differentially expressed miRNAs showed a preference for gametophores with sporophytes (Fig. 1e), suggesting that they function mainly during the adult gametophyte or sporophyte stage (Table 1). Ppt-miR1028c-3p, which is highly specific to this stage ($\sim$38-fold increase), guides the cleavage of an mRNA that encodes a protein with strong similarity to the *A. thaliana* protein arginine N-methyltransferase 4a (AtPRMT4a) (Addo-Quaye et al. 2009). Recombinant AtPRMT4a can asymmetrically dimethylate histone H3 (Niu et al. 2008). Loss of function of AtPRMT4a and AtPRMT4b caused elevated levels of FLOWERING LOCUS C (FLC) mRNA, indicating that AtPRMT4a and AtPRMT4b affect flowering time by repressing FLC probably at the epigenetic level (Niu et al. 2008). Thus, Ppt-miR1028c-3p may negatively regulate *PpPRMT4a* and as a result activate gene expression in the mature moss gametophyte or sporophyte.

miRNA targets with putative transcription factor functions

In *A. thaliana*, a high proportion of mRNAs, which are cleaved by conserved miRNAs, encode transcription factors, while targets of specific miRNAs have more diverse functions (Fahlgren et al. 2007). Surprisingly, almost half of the *P. patens* validated targets encode putative DNA-binding transcription factors, many of which are targeted by specific miRNAs (Addo-Quaye et al. 2009; Arazi et al. 2005; Axtell et al. 2007; Talmor-Neiman et al. 2006a), suggesting that miRNA regulation of gene expression programs in moss may be more common than in flowering plants. In addition, many of the transcription factor targets are members of families that have been shown to be involved in developmental regulation, response to stress or both in flowering plants implying that miRNAs function as master regulators of development and stress adaptation in *P. patens*.

A fraction of these are targeted by conserved miRNAs in *P. patens* and flowering plants (Addo-Quaye et al. 2009; Arazi et al. 2005; Axtell et al. 2007). They include members of the SQUAMOSA Promoter Binding Protein (Ppt-miR156), ARF (Ppt-miR160), class-III HD-ZIP (Ppt-miR166), GRAS-domain (Ppt-miR171) and MYB (Ppt-miR319) families of transcription factors, with demonstrated functions in development in *A. thaliana* (Chen 2009; Wang et al. 2010). The conservation of these miRNA-target regulons in *P. patens*, an organism with different morphology and life cycle is intriguing. It was found that several key developmental gene families in flowering plants have conserved functions in *P. patens*, while others have been recruited for newly evolved developmental innovations (Floyd and Bowman 2007). Thus, a likely possibility is that at least some of these miRNA:target pairs represent ancient modules that fulfill basic regulatory functions essential for the development or adaptation of land plants.

Several transcription factors that are targeted by specific *P. patens* miRNAs belong to families that are regulated by distinct unrelated miRNAs in seed plants (Chen 2009). These encode members of the AP2/EREBP (Ppt-miR529) and petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2 (NAC)-domain (Ppt-miR1218 and Ppt-miR1223) families, involved in development as well as in stress signal transduction (Olsen et al. 2005; Riechmann and Meyerowitz 1998), and MADS/K-box (Ppt-miR538), ARF (Ppt-miR1219) and helix-loop-helix (HLH) (Ppt-miR902 and Ppt-miR1026) families. *PPM1* encodes one of three MADS box transcription factors that are targeted by the gametophore abundant Ppt-miR538 (Table 1). *PPM1* antisense knock-down plants displayed abnormal sporophyte development and altered leaf morphogenesis (Singer et al. 2007) indicating *PPM1* requirement and the possible involvement of Ppt-miR538 in these processes. Abscisic acid (ABA) is a regulator of abiotic stress responses in flowering plants (Verslues and Zhu 2005) and *P. patens* (Frank et al. 2005b; Khandelwal et al. 2010). Moreover, ABA treatment was found to cause the upregulation of certain drought induced *Phaseolus vulgaris* miRNAs (Arenas-Huertero et al. 2009). It was found that in the presence of ABA, Ppt-miR1026 is induced and its basic HLH transcription factor target (*PpbHLH*) is downregulated, consistent with the presence of its Ppt-miR1026-guided cleavage product and methylation of its genomic DNA (Khraiwesh et al. 2010). These findings suggest that Ppt-miR1026-mediated downregulation of *PpbHLH* may play a role in the adaption to ABA-mediated stress responses.

Additional *P. patens* specific miRNAs target members of LIM-domain containing (Ppt-miR1043), SFP1-domain containing (Ppt-miR1065), STOP1 (Ppt-miR1023) and dehydration-responsive element-binding (DREB) (Ppt-miR1029) transcription factor families that are not known to be posttranscriptionally regulated in flowering plants (Addo-Quaye et al. 2009; Axtell et al. 2007). In *A. thaliana*, STOP1 regulates the signal transduction pathway of proton and aluminum tolerance in roots (Iuchi et al. 2007), and DREB1 and DREB2 transcription factors regulate the signal-transduction pathways under low temperature and dehydration, respectively (Agarwal et al. 2006). This raises the possibility that their *P. patens* homologs and corresponding miRNAs are also involved in stress adaptation.

miRNA targets with additional regulatory functions

Additional validated miRNA targets with putative regulatory functions include BOP-like proteins (Ppt-miR534a), F-BOX proteins (Ppt-miR536), protein kinases (Ppt-miR898, Ppt-miR1078 and Ppt-miR1215) (Addo-Quaye et al. 2009), the histone modifiers arginine N-methyltransferase (Ppt-miR1028c-3p) and histone deacetylases (Ppt-miR1038-5p) and AtCKI1/2-like cytokinin receptor (Ppt-miR1221) (Talmor-Neiman et al. 2006a). The latter suggests the involvement of Ppt-miR1221 in cytokinin-mediated signal transduction. Cytokinin induces bud formation during the transition from juvenile to adult gametophyte in *P. patens* (Schumaker and Dietrich 1998), implicating Ppt-miR1221 in this process.

Autoregulation of miRNA biogenesis

It was demonstrated that miR162 directs *AtDCL1* mRNA cleavage (Xie et al. 2003). In addition, *AtDCL1* pre-mRNA levels might be reduced by the AtDCL1-mediated release of miR838 from its precursor, which is encoded by *AtDCL1* intron 14 (Rajagopalan et al. 2006). Thus, both miR162 and miR838, which depend on AtDCL1 for their maturation, might limit *AtDCL1* expression, suggesting that miRNA biogenesis is subject to negative-feedback regulation in *A. thaliana*. A miRNA targeting *PpDCL1a* has not been identified in *P. patens*. Nevertheless, Ppt-MIR1047 was found to reside in intron 7 of the *PpDCL1a* gene and evidence for DCL1-like Ppt-miR1047/Ppt-miR1047* excision from *PpDCL1a* pre-mRNA was obtained (Axtell et al. 2007), raising the possibility that *P. patens* miRNA biogenesis is subject to negative-feedback regulation similar to *AtDCL1* by miR838.

A. thaliana AGO1 (*AtAGO1*) is negatively regulated by miR168 (Rhoades et al. 2002; Vazquez et al. 2004), which is itself stabilized upon incorporation into the AtAGO1-RISC (Vaucheret et al. 2006). This autoregulatory loop is required for AtAGO1 homeostasis and because AtAGO1 is the major miRNA slicer enzyme (Baumberger and Baulcombe 2005; Qi et al. 2005), it is also required for proper functioning of the miRNA pathway and normal

development (Vaucheret et al. 2004). *P. patens* has three AGO1-like genes (*PpAGO1a-c*). These genes were found to be targeted for cleavage by the moss-specific Ppt-miR904, suggesting that it regulates their levels in a manner reminiscent of miR168 regulation of *AtAGO1* (Axtell et al. 2007).

The negative regulation of *AGO1* and *DCl1* genes by distinct miRNAs in mosses and angiosperms emphasize the importance of these autoregulatory loops for miRNA functions and suggest the occurrence of convergent miRNA evolution.

Regulation of the juvenile-to-adult gametophyte transition by miRNAs

Studies have revealed that miR156, miR172 and miR390 are involved in the regulation of developmental timing in flowering plants (Fahlgren et al. 2006; Poethig 2009). In *P. patens*, miR390 directs the cleavage of *PpTAS1-4*, enabling the production of phased trans-acting siRNAs (ta-siRNAs) that target *ARF3*, *ARF4* and several AP2-like genes (Axtell et al. 2006; Axtell et al. 2007; Talmor-Neiman et al. 2006b). *P. patens* RNA-dependent RNA polymerase 6 (*PpRDR6*) is required for ta-siRNAs production (Talmor-Neiman et al. 2006b). A Δ*PpRDR6* mutant that does not express ta-siRNAs exhibited an accelerated transition from juvenile to adult gametophyte implicating miR390 in the regulation of this process in moss (Talmor-Neiman et al. 2006b). Interestingly, in the *A. thaliana* sporophyte, distinct miR390-dependent *TAS3* ta-siRNAs, which also target *ARF3* and *ARF4* (Allen et al. 2005), are required for vegetative phase transition (Adenot et al. 2006; Fahlgren et al. 2006), raising the possibility that regulation of developmental timing by miR390 was conserved during land plant evolution. Nevertheless, Ppt-miR390 was also found to direct the cleavage of two mRNAs encoding unknown proteins (Addo-Quaye et al. 2009) and hence may be involved in additional processes in moss.

The protonema-specific Ppt-miR534a (Table 1)(Arazi et al. 2005) guides the cleavage of *PpBOP1* and *PpBOP2* (Addo-Quaye et al. 2009; Axtell et al. 2007). PpBOP1 and PpBOP2 encode homologs of *A. thaliana* BOP1 & BOP2, which function as transcriptional activators that promote cell differentiation events (Jun et al. 2010; McKim et al. 2008; Xu et al. 2010). Deletion of *PpMIR534a* caused elevated *PpBOP1/2* transcript levels and accelerated gametophore development, thus correlating high *PpBOP1/2* levels with premature bud formation (Saleh et al. 2011). Consistent with this, exposure to cytokinin, which induce bud formation (Schumaker and Dietrich 1998), down regulated *Ppt-MIR534a* transcription and increased PpBOP1 accumulation in apical caulonema cells. Reporter gene fusions showed that *PptMIR534a* is ubiquitously expressed in protonemata whereas PpBOP1/2 accumulation is restricted almost exclusively to potent caulonema apical cells and their side branch initials, but is absent from differentiated cells. These data suggest a conserved role for *PpBOP1/2* in promoting cell differentiation and show that *Pp-miR534a* is required for control of the juvenile-to-adult gametophyte transition via regulation of the spatial and temporal expression of *PpBOP1/2* in protonema cells (Saleh et al. 2011).

miRNA-mediated transitive and epigenetic silencing of target genes

In *A. thaliana*, miRNA-associated transitivity, namely the production of small interfering RNAs (siRNAs) from regions upstream or downstream of a miRNA target site, was observed for few targets (Howell et al. 2007; Luo et al. 2009) and miRNA-dependent methylation at target gene loci has been demonstrated once for miR165/166 and its target genes *PHABULOSA* and *PHAVOLUTA* (Bao et al. 2004). A recent study suggests that both silencing pathways exist and may be more prevalent in *P. patens* (Khraiwesh et al. 2010). Small RNA gel blots probed with sequences that flank the miRNA target site of *PpARF* and *PpC3HDZIP1*, which serve as targets for miR160 and miR166, respectively, detected the presence of sense and antisense transitive siRNAs indicating spread of silencing along the miRNA target in both 5′-3′ and 3′-5′ directions. In addition, transitive siRNAs were not detected in Δ*PpDCL1b* mutants that were compromised for miRNA-mediated target cleavage suggesting their dependence on target cleavage (Khraiwesh et al. 2010). Although miRNA-mediated target cleavage was abolished in Δ*PpDCL1b* mutants, target levels were reduced due to hypermethylation of their genomic sequences. Moreover, in wild-type *P. patens*, target locus methylation was directly correlated with relatively high miRNA levels induced by either ectopic expression of an artificial miRNA or ABA treatment under physiological conditions. In all of these cases, stable miRNA:target duplexes were detected and were associated with target-gene methylation. A model was proposed in which free miRNAs, which may be abundant in Δ*PpDCL1b* mutants with a non-functional RISC or due to high expression, promote the formation of miRNA:target duplexes that trigger epigenetic silencing of target genomic sequences (Khraiwesh et al. 2010).

Approaches to studying miRNA functions in *P. patens*

The cloning of the lion's share of *P. patens* miRNAs, their expression profiling by deep sequencing during major

stages of development and the experimental validation of many of their target RNAs provide a framework for the elucidation of their various biological functions in this model organism.

Analysis of a loss-of-function miRNA mutant is an important prerequisite to understanding that miRNA's precise biological function. Contrary to flowering plants for which only a few miRNA loss-of-function mutants have been isolated (Allen et al. 2007; Baker et al. 2005; Beauclair et al. 2010; Williams et al. 2005), generation of loss-of-function mutants in *P. patens* is a simple task. This is due to this organism's ability to perform efficient gene targeting (Schaefer 2001) and to the availability of its genome sequence (Rensing et al. 2008), which is expected to streamline the design of *MIR* gene disruption constructs containing homologous genomic sequences. So far, sequential knockout of up to three genes using selection markers that confer resistance to geneticin, hygromycin and zeocin has been demonstrated in *P. patens* (Khandelwal et al. 2010; Sakakibara et al. 2008). Thus, a reverse-genetics approach to deciphering the functions of 89 out of 106 miRNA families with three or less members should be feasible. Nevertheless, gene disruption of certain multi-member miRNA loci may also be informative if the disrupted *MIR* locus is a major contributor to miRNA levels or drives specific expression at a certain developmental stage or in a specific tissue. For example, disruption of *PpMIR534a*, one of two Ppt-miR534 encoding loci that express the majority of Ppt-miR534 (Axtell et al. 2007), reduced its expression dramatically and increased the levels of its *PpBOP1/2* target genes (Saleh et al. 2011). In addition, it was recently demonstrated that an artificial miRNA designed against a mature miRNA sequence is able to knockdown all miRNA family members (Eamens et al. 2010). A similar approach can be used for the functional analysis of the remaining 17 multi-member miRNA families in *P. patens* (Axtell et al. 2007), since an artificial miRNA, based on the *A. thaliana* pre-miR319a, has already been shown to silence specific genes in this moss (Khraiwesh et al. 2008). An alternative method may be to express target mimics against miRNA families since those could prevent them from silencing their targets in *A. thaliana* (Franco-Zorrilla et al. 2007; Todesco et al. 2010). Generated loss-of-function miRNA mutants and mutants altered in the production of a particular miRNA can be analyzed for their phenotype to reveal deviations from the wild type under different physiological conditions. In addition, whole-transcriptome analysis of these mutants would provide insight into the pathways and gene-expression networks affected by the absence of a specific miRNA and might identify novel target RNAs. Cleavage and degradome analyses revealed the RNA targets of one-third of identified *P. patens* miRNA families (Addo-Quaye et al. 2009; Arazi et al. 2005; Axtell et al. 2007; Fattash et al. 2007). It was postulated that miRNA-mediated translational inhibition might be widespread in plants (Brodersen et al. 2008). This raise the possibility that proteome analysis, an established technique in *P. patens* (Sarnighausen et al. 2004), of miRNA loss-of-function mutants might be useful in identifying targets whose translation may be inhibited by the corresponding miRNA.

To fully understand a miRNA's function, its regulatory role needs to be deciphered and the biological significance of its repression needs to be determined for an individual miRNA/target pair. To understand the regulatory role of a miRNA, one needs to determine its expression pattern and how the expression pattern of its target RNA is modified upon its release from miRNA regulation (miRNA resistance). The biological significance of miRNA-based repression can be elucidated by expressing both wild-type and miRNA-resistant target forms under their native promoter in transgenic plants and comparing the resultant phenotypes (Garcia 2008). The high frequency of homologous recombination in *P. patens* (Schaefer and Zryd 1997) is very useful in both cases, because it enables precise sequence replacement while maintaining the original genomic context (Cove 2005). A gene-replacement construct can be designed to replace the original target gene sequence with a mutated miRNA-resistant form in order to study phenotypic consequences in transgenic plants. A gene-replacement construct can also be used to fuse a reporter gene to a wild-type or miRNA-resistant target gene locus in order to monitor their authentic accumulation pattern in the presence or absence of miRNA regulation, respectively.

In conclusion, *P. patens* extraordinary ability, among land plants, to perform efficient gene targeting provides a unique opportunity to elucidate the functions of all miRNAs in a land plant. Applying the above approaches to study the deeply conserved and specific *P. patens* miRNAs is expected to reveal conserved as well as novel functions of these regulators some of which are expected to provide insights into land plant evolution and how early land plants adapted to life on land.

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