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MicroRNA-mediated establishment of transcription factor gradients controlling developmental phase transitions

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The juvenile-to-adult phase transition is an important and critical step during plant development to ensure maximum reproductivity. This transition is regulated by different pathways, in some of which microRNAs are considered to be essential key components. In seed plants, miR156 and miR172 act sequentially in well characterized pathways to induce the vegetative phase change and floral formation by the establishment of spatiotemporal gradients of their cognate target transcripts that encode master regulators of development. Recently, we reported on an unrelated, moss-specific miRNA that acts similarly in the control of the juvenile-to-adult phase transition in *Physcomitrella patens*. Physcomitrella miR534a defines the spatial expression of two transcripts encoding BLADE-ON-PETIOLE (BOP) transcriptional coactivators in a cytokinin-dependent manner. We propose that this miRNA-mediated control is a major mechanism underlying the cytokinin-induced formation of the gametophore meristem in Physcomitrella. Furthermore, it suggests a convergent evolution of miRNA-controlled pathways regulating phase transitions in seed and non-seed plants.

Key words: development, phase transition, microRNA, BLADE-ON-PETIOLE, cytokinin, *Physcomitrella patens*

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Completion of a plant's life cycle requires the transition between different developmentally distinct phases. This includes the vegetative, non-reproductive juvenile to vegetative adult transition, referred to as the vegetative phase change,[1] and the successive switch to a reproductively competent adult. The coordination of the transition onset between these phases with environmental cues is important to ensure maximum reproductivity. The regulation of these developmental transitions occurs through different pathways, some of which are dependent on environmental shifts while others are at least partially environmental cue-independent and termed as "autonomous pathways."[2]

In recent years, several key players of these pathways were identified including microRNAs (miRNAs), a specific class of nuclear-encoded ~21 nucleotides small, non-coding RNAs that bind to cognate target RNAs by sequence complementarity[3] and negatively regulate target expression by mediating mRNA cleavage,[4] translational inhibition,[5] or transcriptional repression.[6] Various plant miRNA families and their corresponding RNA targets are highly conserved between different plant species implying evolutionary conserved functions, whereas others are less conserved or species-specific. Many plant miRNAs have pivotal functions in the control of various biological processes such as development, cell differentiation, phytohormone signaling, apoptosis, biotic and abiotic stress responses.[7,8] A regulatory function of miRNAs in the control of the juvenile-to-adult transition was recently reported for the seed plants *Arabidopsis thaliana* and maize,[9] and also suggested to be present in rice.[10]

In angiosperms a regulatory network involving the conserved miRNAs miR156,[11] and miR172,[12] was found to be a master regulator of autonomous pathways controlling the vegetative phase change and flowering time, respectively (**Fig. 1A**).[2,9] miR156 negatively regulates mRNAs

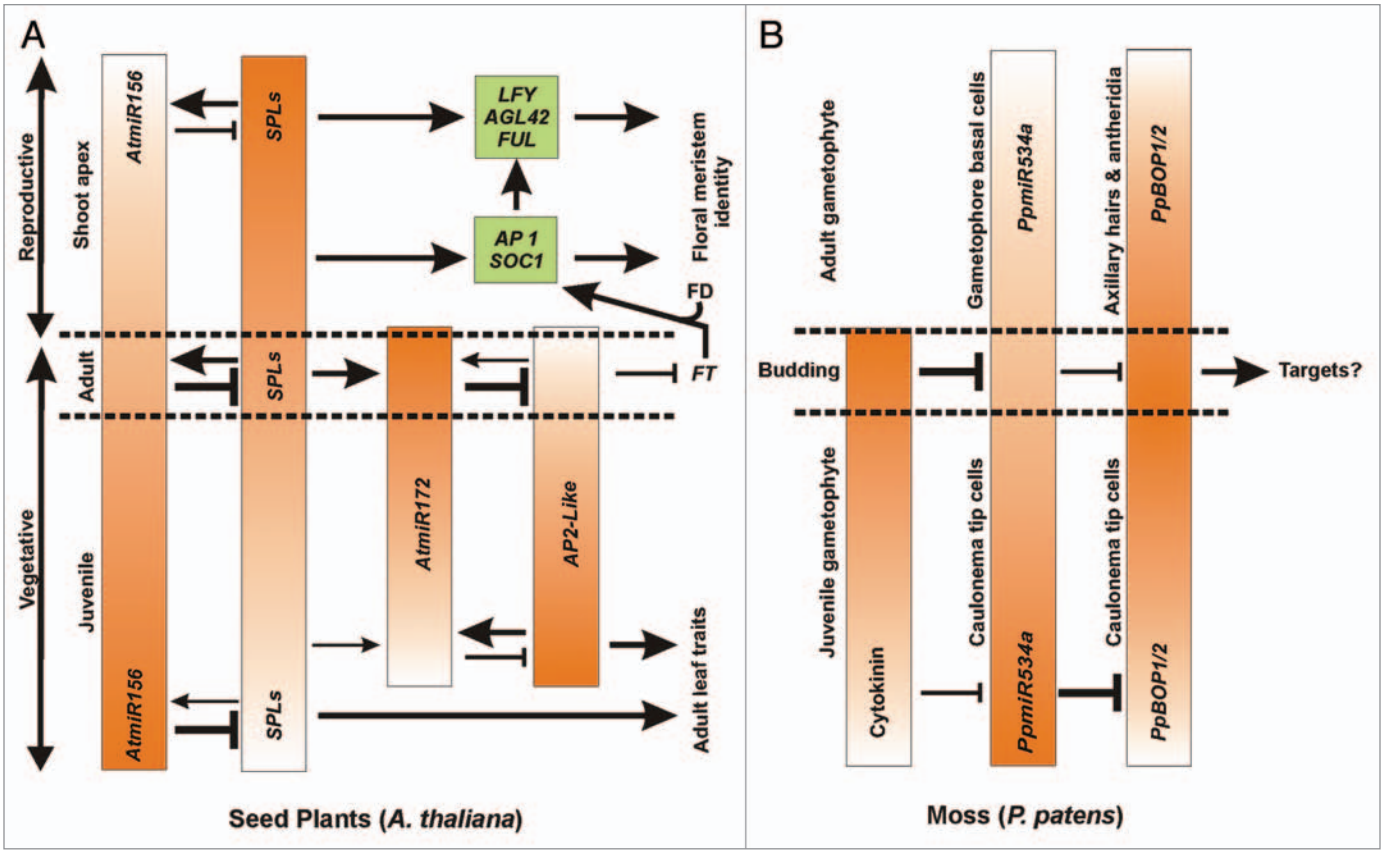

Figure 1. Comparison of miRNA-mediated juvenile-to-adult transition pathways in (A) seed plants (adopted from Chen et al.[10]) and (B) moss. Vertical color gradients indicate gradual gene expression at particular developmental stages or spatially restricted gene expression in particular cells or tissues (dark color, high expression; light color, low expression). Arrows: positive regulation; bars: negative regulation. See text for full gene names.

encoding SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors,[11,13] some of which, SPL9 and SPL10, directly activate the transcription of miR172.[14] In turn, miR172 targets and downregulates transcripts encoding APETALA2 (AP2)-like repressors of the central flowering-inducer gene *FLOWERING LOCUS T (FT)*.[15,16] FT is transported from leaves to the shoot apex where it forms a complex with the bZIP transcription factor FD to activate the floral MADS-box genes *APETALA1* (*API*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*).[17] Other miR156 *SPL* targets (SPL3, SPL4 and SPL5) are activated at the shoot apex and regulate, in an miR172 and FT-independent manner, the expression of *API*, *SOC1* and additional flowering-related factors such as *LEAFY* (*LFY*), *FRUITFULL* (*FUL*), and *AGAMOUS-LIKE 42* (*AGL42*).[2,18,19] Beside floral induction, the miR156-SPL-miR172-AP2 regulatory networks mediate leaf trait shifts during the vegetative phase change from juvenile-to-adult traits in Arabidopsis.[10,14,20] Thus, these regulatory networks require a strict control of *SPL* and miR172 expression in the early phases of development to maintain juvenility and to avoid precocious onset of adult traits and flower formation.[18] This control is released throughout development by the gradual decrease of miR156 and the synchronous increase of the *SPL* and miR172 that trigger juvenile-to-adult transition (**Fig. 1A**).[10,14,20]

These findings raise the question about the internal signals that act upstream of such molecular networks. Phytohormones were reported to act as internal signals regulating phase transitions. For example, gibberellins promote floral formation and cone production in Arabidopsis and conifers, respectively.[21,22] Gibberellin-induced promoter activation of the floral meristem identity gene *LFY* was reported as one mechanism by which gibberellins promote flowering in Arabidopsis.[21] However, in woody angiosperms gibberellins act conversely by promoting vegetative growth and inhibiting flowering.[22] Recent evidences also link cytokinins with floral induction in Arabidopsis via the activation of the *FT* paralogues *TWIN SISTER OF FT* (*TSF*) and *SOC1*.[23] Wang et al.[18] tested whether different phytohormones including gibberellins, auxins and cytokinins affect *AtmiR156* expression levels in whole seedlings, but observed no obvious effect. These findings indicate the importance of phytohormones in the regulation of the juvenile-to-adult transition in seed plants, but we still await direct evidence that links phytohormone action to miRNA-based regulatory pathways in this process.

Cytokinins are known to promote the juvenile-to-adult transition in mosses, e.g., *Physcomitrella patens*.[24] The life cycle of Physcomitrella is dominated by a haploid gametophytic generation that is characterized by two distinguishable developmental

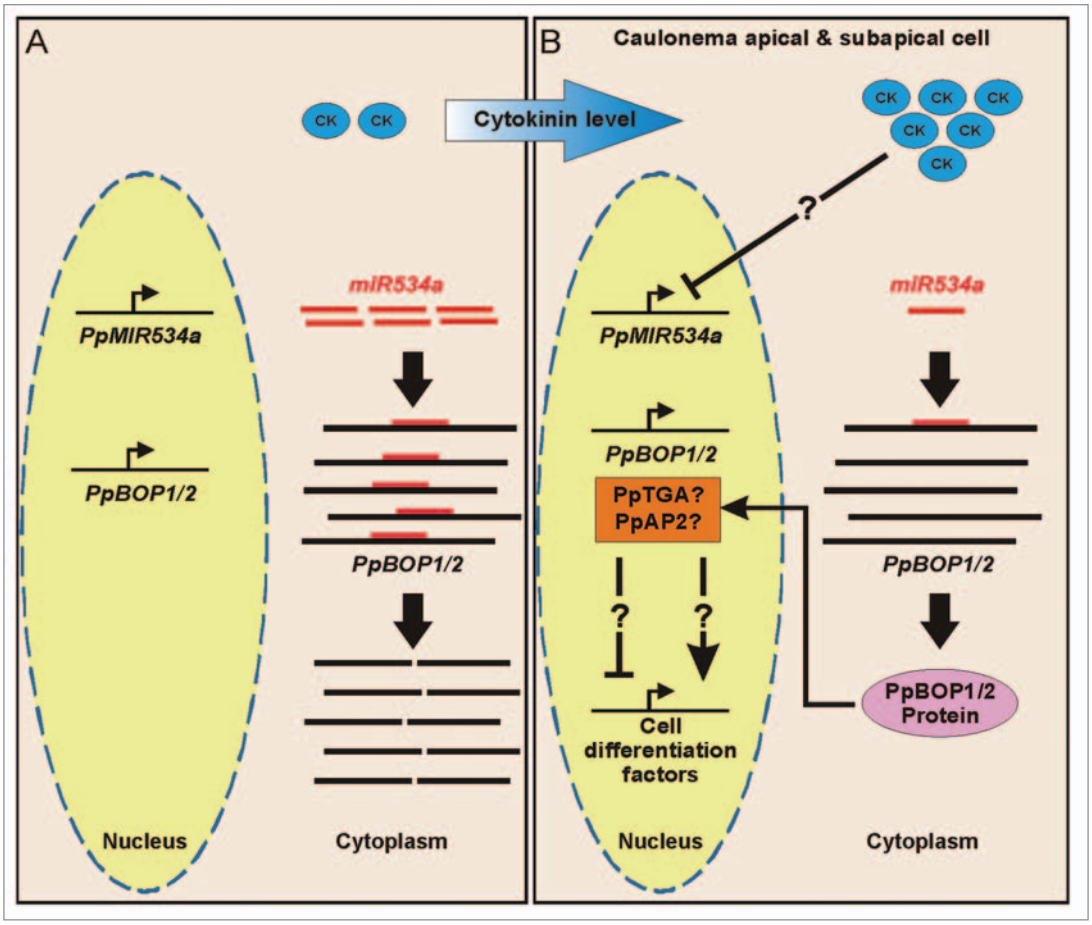

Figure 2. Model of the cytokinin-dependent *PpMIR534a-PpBOP1/2* regulatory network controlling the juvenile-to-adult transition in Physcomitrella. (A) During early development *PpMIR534a* is expressed within all protonema cells causing effective cleavage of *PpBOP1/2* transcripts. The low endogenous cytokinin (CK) levels at this developmental stage cannot inhibit *PpMIR534a* expression. (B) A gradual increase of endogenous cytokinin along the protonema filament causes repression of *PpMIR534a* via an unknown mechanism in caulonema apical and subapical cells. Repression of *PpMIR534a* leads to reduced miR534a levels and less efficient *PpBOP1/2* target cleavage promoting the accumulation of PpBOP1/2 transcriptional coactivators that may interact with TGA and/or AP2-domain transcription factors to control the expression of cell differentiation factors that act in bud meristem formation.

phases: the juvenile filamentous protonema and the subsequently emerging adult leafy gametophore that upon maturation harbors both male and female sex organs.[25] Protonema filaments proliferate by apical cell divisions whereas the transition to the adult gametophore that is trigged by cytokinin in a concentration-dependent manner requires the formation of a three-faced apical cell giving rise to a bud meristem.[24] However, in contrast to seed plants the molecular mechanisms underlying this vegetative phase transition are not yet characterized. Previous studies on miRNAs in Physcomitrella revealed a development-dependent differential expression of some miRNAs pointing to a possible role of these regulatory elements in phase transitions in this species.[26,27]

We recently identified a network regulating the juvenile-to-adult transition in Physcomitrella consisting of cytokinin and the species-specific miR534a along with its target transcripts encoding homologues of the Arabidopsis transcriptional coactivators BLADE-ON-PETIOLE 1 and 2 (AtBOP 1/2) (**Figs. 1B and 2**).[28] These findings provided first evidence for a regulatory role of miRNAs in the regulation of developmental phase transitions in non flowering plants.

Targeted disruption of the *PpMIR534a* genomic locus in Δ*PpMIR534a* mutant lines resulted in the loss of miR534a accompanied by an accelerated bud formation when compared to Physcomitrella wild type plants.[28] miR534a targets *PpBOP* transcripts by mediating mRNA cleavage of two out of three members of this small gene family, namely *PpBOP1* and *2*. Due to the lack of miR534a and abolished *PpBOP1* and *2* target cleavage steady-state expression levels of both targets were increased in Δ*PpMIR534a* mutant lines. Elevated *PpBOP1/2* transcript levels in the Δ*PpMIR534a* mutants lead to precocious bud formation by promoting cell differentiation into three-faced apical cells. This defect in the timing of bud formation in Δ*PpMIR534a* mutants by the deregulation of the miR534a-dependent regulatory network suggests that *PpBOP1/2* expression in Physcomitrella wild type is strictly regulated by miR534a to define a temporal and/or spatial expression pattern that underlies the proper onset of bud formation. *PpMIR534a*-promoter::*GUS*-fusions

revealed an ectopic expression of miR534a throughout juvenile protonema filaments and young developing buds. However, the expression of miR534a-sensitive *PpBOP*::GUS fusion constructs targeted to the corresponding *PpBOP1* and *2* genomic loci, and thus, were controlled by the endogenous promoters, was restricted to apical and subapical caulonema cells of the filaments. Based on these results we hypothesized that the specific spatial expression of *PpBOP1* and *2* is controlled by varying expression levels of miR534a within a protonema filament. In the basal regions of a protonema filament miR534a expression is sufficient to clear *PpBOP1/2* expression whereas miR534a expression levels in apical and subapical caulonema cells might be gradually repressed leading to increased *PpBOP1/2* expression within these cells. Thus, miR534a may act as a buffer system that is able to control *PpBOP1*and *2* expression levels within specific cells to prevent overaccumulation of *PpBOP1* and *2* transcripts and subsequent bud formation (**Fig. 2**).

Since the phytohormone cytokinin triggers the vegetative phase transition and bud formation in Physcomitrella, the miR534a-*PpBOP1/2* regulatory network might be a master regulatory switch acting downstream of cytokinin. Treatment of protonema filaments with cytokinin caused a specific repression of *PpMIR534a* in apical and supapical caulonema cells throughout the entire Physcomitrella colony. In addition, PpMIR534a repression within these cells was accompanied by an increased PpBOP1 and 2 expression indicating the presence of a miR534a-PpBOP1/2 regulatory system operating in a cytokinin-dependent manner within these cells (**Fig. 2B**).

It is known that cytokinin increases gradually during Physcomitrella development reaching its maximum immediately before the onset of budding.[24] Thus, it is likely that this gradual elevation of endogenous cytokinin will lead to a gradual decrease of miR534a expression specifically in the undifferentiated apical and subapical cells via a so far unknown pathway. Possible scenarios include a cytokinin-responsive repression of transcriptional regulators that activate the *PpMIR534a* promoter and/or cytokinin-mediated induction of transcriptional repressors that downregulate *PpMIR534a* promoter activity. This cytokinin-dependent gradual downregulation of *PpMIR534a* within these cells causes accumulation of *PpBOP1* and *2* that reach certain threshold levels to trigger bud formation (**Fig. 2B**).

So far, the downstream targets of the miR534a-controlled PpBOPs remain unknown. Arabidopsis BOP proteins act as transcriptional coactivators in the control of lateral organ architecture by promoting cell differentiation in their proximal regions.[29-32] AtBOP1 and 2 form homo-oligomers within the cytosol, but interact with TGA transcription factors, e.g., PERIANTHIA (PAN) upon translocation to the nucleus[29] to induce the expression of factors promoting cell differentiation such as *ASYMMETRIC LEAVES2* (*AS2*) and *APETALA1* (*API*) at the leaf base and floral shoot, respectively.[31,32] Beside controlling lateral organ morphogenesis, first evidence for a role of AtBOPs in the vegetative-to-reproductive phase transition was provided by Karim et al.[33] since they identified overlapping actions of AtBOPs and AtPUCHI in promoting expression of *LFY* and *API*, both being essential regulators of floral meristem identity. Whether PpBOPs act in similar pathways remains to be shown. However, the characterization of PpBOPs revealed a high similarity to AtBOPs with regard to protein structure and their dual subcellular localization in the cytoplasm and the nucleus. Furthermore, the genome of Physcomitrella encodes several close homologues of AtBOP-interacting proteins (AtPAN, AtTGA and AtPUCHI) and their downstream targets (AtAS2, AtAP1, AtLFY) suggesting similar modes of action of BOP proteins in mosses and seed plants.[28] Further analyses are required to elucidate the genes participating in this pathway, especially those acting upstream of miR534a and downstream of PpBOPs. Moreover, it remains open whether other miRNA-controlled regulatory networks might be involved in the vegetative phase transition in Physcomitrella. The miR156-SPL regulon that directly activates the miR172-AP2-FT network to induce vegetative phase transition and floral induction in Arabidopsis seems to be incompletely conserved in Physcomitrella. Even though miR156 and its cognate *SPL* target transcripts are conserved, miR172 seems to be lacking in Physcomitrella.[26,34] The differences in mosses and seed plants, however, suggest a convergent evolution of miRNA-controlled pathways that are indispensable for the control of developmental switches during plant growth.

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