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Time to Grow up: the Temporal Role of smallRNAs in Plants

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Abstract

Over the past two years, several *Arabidopsis* genes that were initially identified as vegetative phase change mutants have been shown to have roles in smallRNA (sRNA) biogenesis. This has led to the identification of a new class of short interfering RNAs (siRNAs) called trans-acting siRNAs (ta-siRNAs).

Introduction

Since their initial discovery in 1993, smallRNAs (sRNAs) have proven to be important biological regulators. These 19-24-nt RNAs consist of two classes, microRNAs (miRNAs) and short interfering RNAs (siRNAs), which differ in their biogenesis and functions [1,2]. miRNAs characteristically regulate non-self transcripts, those with homology within the miRNA binding site but limited sequence homology to the rest of the miRNA precursor, whereas siRNAs usually target only highly homologous genes [1,3].

miRNAs have various functions in animal and plant development. To date, plant miRNAs have been shown to influence leaf morphology, polarity, floral organ identity, organ fusion, and even stress responses, and many other functions have been postulated on the basis of their putative target genes [3,4,5*,6*,7-9,10*,11]. By contrast, siRNAs are important for mediating transcriptional gene silencing (TGS) of repeated sequences, such as transposons and heterochromatic repeats, and the post-transcriptional gene silencing (PTGS) of RNA from exogenous sources, such as viruses and transgenes [2,12].

The discovery that sRNAs could affect developmental transitions first came from studies in *Caenorhabditis elegans* showing that *lin-4* and *let-7* encode temporally regulated miRNAs that translationally inhibit the expression of several heterochronic genes [13,14]. Since that time, sRNAs have also been shown to regulate temporal changes in *Drosophila* [13], and recent studies in *Arabidopsis* suggest that they have a similar function in plants [15,16*,17*,18*]. Here, we briefly review the field of vegetative phase change, highlight older literature from several fields which suggest that RNA-silencing activities might be correlated with phase change, and discuss recent data that demonstrate a role for sRNAs in this transition.

Vegetative phase change

Flowering plants pass through three primary postembryonic developmental stages: juvenile, adult, and reproductive. The transition from the adult to the reproductive phase (floral

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Note added in proof

Since the submission of this manuscript, two papers have been published that demonstrate the importance of ta-siRNAs in the regulation of ARF (AUXIN RESPONSE FACTOR) genes [52**,53]. Allen et al.[52**] also show that miRNAs can target ta-siRNA precursors, regulating ta-siRNA phasing. Because miRNA biogenesis requires DCL1, this probably explains the observation that ta-siRNA production is reduced in *dcl1* mutants [17**,18**,52**,53].

transition) is quite obvious, and has been studied intensively. Less is known about the juvenile-to-adult transition. Distinct juvenile and adult vegetative phases were first described and are most obvious in woody plants, such as *Hedera helix* [19,20], but they are also evident in herbaceous plants, such as maize and *Arabidopsis* [21].

In *Arabidopsis*, some factors, such as leaf shape and the numbers of hydathodes and adaxial trichomes, change on a continuum throughout vegetative growth. Other traits are specific to one phase or the other. The juvenile phase is characterized by leaves that are fairly round with smooth margins, a relatively low blade-to-petiole ratio, and no abaxial trichomes [15,22-24]. By contrast, leaves of the adult phase are ovate and have serrate margins, a relatively shorter petiole, edges that curl downward, and abaxial trichomes. The full shift from juvenile to adult occurs over several leaves. Leaves produced during this transition are composites of juvenile and adult tissue, with the proximal portion displaying more adult characteristics and the distal portion being more juvenile [22,23]. The chimeric nature of these leaves is explained by the fact that vegetative phase change is initiated by unknown factors that are produced outside the meristem and that act directly on competent individual leaf primordia rather than on the shoot apical meristem [21].

Analyses of factors that affect the juvenile-to-adult and reproductive transitions in various species have shown that these transformations are coordinated and yet distinct. Gibberellic acid (GA), phytochrome B, and vernalization, as well as other unknown factors, regulate the onset of both phases, and thereby probably coordinate these two transitions [21-23,25]. Some mutations alter both the length of the juvenile phase and the time to flowering, further demonstrating that common factors regulate these processes [22,23,25-28]. However, many of the signaling pathways and mutations that affect flowering time do not have a role in vegetative phase change [21,25,29-31]. For example, the *terminal flower1-10* (*tfl1-10*) mutant flowers early but its vegetative phase change is unaffected [23]. Further, mutants that have an early adult phase transition do not necessarily flower earlier [15,17^{**},32].

The most interesting mutants for understanding vegetative phase change specifically affect the juvenile-to-adult phase transition but not reproductive competence. These include the *serrate* (*se*) mutant, which has a defect in a single zinc finger gene [33,34]; the *squint* (*sqn*) cyclophilin 40 mutant [32]; the *zippy* (*zip*; also known as *argonaute7* [*ago7*] [15]) mutant, which has defects in an AGO family member; and two mutants that have defects in genes that are required for PTGS, *RNA-dependent RNA polymerase6* (*rdr6*) and suppressor of gene silencing3 (*sgs3*) [17^{**},18^{**}]. The identification of *rdr6* and *sgs3* as phase change mutants is particularly interesting because it suggests a link between vegetative phase change and RNA silencing (see below).

Temporal changes in silencing in plants

Developmental regulation of transposon silencing in maize

RNA-silencing patterns can vary temporally during development. For example, work with two maize transposons, Suppressor-mutator (Spm) and Robertson's Mutator (Mu), has shown that transposon silencing can increase during shoot development [35-39]. siRNAs are important for the establishment of transposon silencing by DNA methylation [40]. Plants that have active Spm or Mu transposons at germination display a gradual decrease in transposon activity and an increase in transposon methylation along the primary shoot of the plant [35-38]. Rudenko et al.[39] further showed that developmentally regulated methylation and silencing are paralleled by a gradual reduction in the polyadenylation of Mu-derived RNA and by an increase in the nuclear retention of these RNAs, resulting in fewer mature transcripts.

The temporal regulation of the Spm and Mu transposons was demonstrated most clearly in studies with the pale green mutant *hcf106* and the lesion mutant *Les28*, whose mutant phenotypes are caused by the activity of Mu transposons [37,38]. Upon inactivation of the corresponding transposons, the phenotypes of these mutants are suppressed, and sectors of wildtype tissue can be seen. These sectors increase in size and number in subsequent lateral organs as the plant develops. As a result, the juvenile leaves tend to have the greatest transposon activity, the adult leaves and ears (containing the female gametophyte) an intermediate activity, and the pollen the lowest activity [37,38]. These changes in epigenetic states, although reversible under the appropriate conditions, are both mitotically and meiotically heritable.

Because the pollen has a lower transposon activity than tissues generated during earlier developmental stages, it has a relatively high chance of passing on an inactive transposon state to its progeny [36-38]. This developmental transposon silencing has not been correlated with phase change and, unlike the phase transitions, is probably a cumulative phenomenon rather than a stepwise one.

Methylation patterns of *Pl-Blotched* in maize are coordinated with vegetative phase change

The methylation patterns of the maize epigenetic allele *Pl-Blotched* are regulated in a highly vegetative-phasespecific manner [41]. *Pl-Blotched* is an allele of the purple plant1 gene, which encodes a key transcription factor for modulating the expression of anthocyanin biosynthetic genes [42]. The *Pl-Blotched* allele expresses a lower level of *pl* mRNA than its probable ancestor *Pl-Rhoades*, resulting in variegation of the plant [42]. Hoekenga et al. [41] found that the *Pl-Blotched* allele is more highly methylated and has a more closed chromatin domain than *Pl-Rhoades*. Further, the level of methylation and the size of the closed chromatin domain increase during the juvenile-to-adult vegetative transition, peaking in adult leaves [41]. Like vegetative phase change, the methylation and chromatin state of this locus are reset each generation. On the basis of these findings, Hoekenga et al. [41] speculated that the developmental regulation of *Pl-Blotched* is controlled by signals that also control vegetative phase change.

Transgene-induced cosuppression is developmentally regulated

The first evidence that PTGS might play a role in phase change came from several reports of transgene-induced cosuppression in *Nicotiana tabacum* and *Arabidopsis* [43-46]. Cosuppression is the siRNA-mediated silencing of transgenes and any identical endogenous genes that is caused by the overexpression of a sense transcript. When under the control of the 35S promoter, transgenes that were expressed in young seedlings often underwent progressive silencing in successive leaves until little or no transcript persisted. Silencing was dependent on the level of gene expression: hemizygous transgenes remained active, whereas homozygous transgenes were silenced [43-46]. As with the inheritance of *Pl-Blotched*, progeny had the same developmentally regulated transgene-silencing pattern as their parents, implying that silencing was reset every generation.

Genes that are important for sRNA biogenesis are involved in phase change

The discovery of a new class of early adult onset *Arabidopsis* mutants in 2003 and 2004 firmly merged the fields of vegetative phase change and sRNAs [15,17^{**},18^{**}]. When the mutated genes were identified, they were found to correspond to the AGO family member *ZIP/AGO7* [15] and the RNA-silencing genes *SGS2/SDE1/RDR6* and *SGS3* [17^{**}]. These mutants are the best examples to date of vegetative-phase-specific mutants because they have early expression of adult traits, including abaxial trichomes, leaf elongation, downward curling, and serrations, but do not have many other unrelated phenotypes [15].

Before the isolation of *rd6* and *sgs3* from this screen, no developmental phenotypes had been assigned to these mutants. This discovery was rather surprising because the endogenous functions of these genes were expected to be in transposon silencing and viral defense, not in development. RDR6 is an RNA-dependent RNA polymerase, and SGS3 is a plant-specific protein that is also required for PTGS [44]. Further experiments showed that RDR6 and SGS3 are necessary for the biogenesis of a new class of siRNAs, called trans-acting siRNAs (ta-siRNAs), which target endogenous non-self genes for cleavage similarly to miRNAs [17[•], 18[•]]. Interestingly, the Dicer-like mutant *dcl1*, which was thought to regulate only miRNA, reduces the level of ta-siRNAs as do *rd6* and *sgs3*, suggesting expanded roles for DCL1 [17[•], 18[•]].

Whether ZIP has a role in ta-siRNA biogenesis is still unknown, but is likely on the basis of the phenotypic similarity of *zip* mutants to *rd6* and *sgs3*. Although ZIP is not required for PTGS, genetic analyses suggest that it is in the same pathway as RDR6 and SGS3 [17[•]]. Other AGO family genes are involved in sRNA biogenesis [47, 48[•], 49]. In fact, the highly pleiotropic mutant *ago1* also has early abaxial trichomes ([48[•]]; RS Poethig, unpublished). Mutations in at least three more loci yield *zip*-like phenotypes (AG Peragine, MR Willmann, RS Poethig, unpublished), and these genes might also prove to regulate sRNA activities.

Recently, it was also shown that the putative nuclear RNA-export receptor exportin 5 gene HASTY (HST) is probably important for the trafficking of many mature miRNAs, but not of siRNAs, from the nucleus to the cytoplasm [16[•]]. *hst* was originally identified as an early phase change mutant, but it also affects the vegetative-to-reproductive transition, and the growth and development of roots, shoots, and flowers [26]. Park et al. [16[•]] showed that miRNAs are exported from the nucleus in a mature, single-stranded form. In a *hst* background, there is a reduction in the accumulation of most miRNAs, suggesting that HST is important for miRNA biogenesis and/or stability. These results also suggested that miRNA exporters in addition to HST exist in *Arabidopsis* [16[•]].

Conclusions

Because a loss of RNA silencing leads to a precocious adult phenotype in *rd6* and *sgs3*, one or more ta-siRNAs are probably required to promote juvenile development. These ta-siRNAs presumably act during the juvenile phase to suppress a factor(s) that leads to the onset of the adult phase. This presents a paradox: the observation that the silencing of *Spm* and *Mu* transposons, *PI-Blotched*, and transgenes increases during shoot development suggests that RNA silencing promotes the expression of the adult phase and not the juvenile phase. One possibility is that phase change is regulated by a temporal change in the transcription of an sRNA precursor rather than by a change in the activity of the silencing pathway. This explanation assumes that the relatively low activity of this pathway during the juvenile phase is sufficient for the processing of this precursor(s). Another possibility is that sRNAs that are generated during the adult phase downregulate the production of juvenile-promoting sRNAs. Finally, a general developmental increase in RNA-silencing activities might initiate a negative feedback response. For example, the level of a juvenile-promoting sRNA could reach a threshold sufficient to silence its precursor before the precursor is transformed into sRNAs.

How broad a role do ta-siRNAs have in plant development? In contrast to the highly pleiotropic phenotypes of miRNA-biogenesis mutants, such as *dcl1* [50, 51], the phenotypes described for *rd6* and *sgs3* are primarily related to vegetative phase change. Thus, it is likely that ta-siRNAs have more limited functions in plant development than do miRNAs. Following the identification of additional ta-siRNAs and their target genes, more specific assays will probably identify other phenotypes of the *rd6* and *sgs3* mutants.

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