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Review

Gene silencing in plants: A diversity of pathways

Angel Emilio Martínez de Alba [1], Emilie Elvira-Matelot [1], Hervé Vaucheret *

Institut Jean-Pierre Bourgin, UMR 1318, INRA, Route de St-Cyr, 78000 Versailles, France

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ABSTRACT

Eukaryotic organisms have evolved a variety of gene silencing pathways in which small RNAs, 20- to 30-nucleotides in length, repress the expression of sequence homologous genes at the transcriptional or post-transcriptional levels. In plants, RNA silencing pathways play important roles in regulating development and response to both biotic and abiotic stresses. The molecular basis of these complex and interconnected pathways has emerged only in recent years with the identification of many of the genes necessary for the biogenesis and action of small RNAs. This review covers the diversity of RNA silencing pathways identified in plants.

1. Introduction

In plants, the natural ability of the pathogen *Agrobacterium tumefaciens* has long been exploited to transfer DNA fragments of known sequence into the genome. However, RNA silencing turned out to be a potential obstacle to transgene reliable expression, in particular when more than one transgene copy is integrated in the genome. Indeed, low transgene expression levels often correlate with high transgene copy number [1,2]. The negative effect of increasing copy number was confirmed when retransforming transgenic plants with a second, partially homologous, transgene [3]. The same effect was observed when adding an extra-copy of an endogenous gene, leading to the so-called co-suppression of transgenes and endogenous genes [4,5]. The extreme sensitivity to copy number was emphasized by the observation that, in many cases, hemizygous transgenic plants expressed the transgene while homozygous siblings were silenced [6,7]. Moreover, promoter strength appeared to play an important role in RNA silencing. Indeed, promoters of viral origin, which have a very high level of expression, triggered transgene silencing more efficiently than weak promoters [8,9]. A comprehensive model involving the production of dysfunctional RNA as a result of aberrant transcription was proposed by Stam and collaborators [10]. Once a critical threshold of dysfunctional RNA would be reached, which is more likely to happen when transgenes are driven by strong promoters, dysfunctional RNA would somehow be transformed into double-stranded (ds)RNA, which is actual trigger of the RNA silencing reaction. The coincidental finding that RNA interference (RNAi) in animals and quelling in fungi also are initiated by dsRNA [11,12] and involve related proteins [13] revealed that RNA silencing likely is an ancient eukaryotic process involved in sequence-specific control of exogenous nucleic acids. Supporting this hypothesis, plants, flies and nematodes use RNA silencing to control viruses by specifically degrading viral RNA [14–16].

RNA silencing also plays essential roles at the endogenous level. Indeed, a large set of endogenous genes, as well as transposons and repetitive genomic sequences, are regulated by RNA silencing, indicating that this mechanism is not only devoted to the control of exogenous nucleic acids, but also to every type of invasive nucleic acids [17,18]. Such regulations are essential during development and reproduction, and serve as a flexible, sequence-specific source of regulation that promotes adaptability in response to biotic and abiotic stresses.

2. Eukaryotes exhibit a diversity of RNA silencing mechanisms

After the discovery of transgene silencing, a rapidly growing number of reports have revealed the extent and diversity of silencing mechanisms controlling exogenous and endogenous sequences. The term "RNA silencing" was created to refer to nucleotide-sequence-specific inhibition pathways mediated by small RNAs. At first, RNA silencing was considered to occur at the transcriptional level (transcriptional gene silencing, TGS), either preventing or dampening transcription through DNA methylation and chromatin modifications, or at the post-transcriptional level (post-transcriptional gene silencing, PTGS) through RNA cleavage or translational repression [18–23]. However, other mechanisms mediated by small RNAs have been recently discovered, including DNA elimination in protists (for review see [24]) or DNA repair in plants, fungi and *Drosophila* [25–28].

Accordingly, the nomenclature of small RNAs also has evolved. At first, small RNAs were divided in microRNAS (miRNAs) and short

* Corresponding author.
E-mail address: herve.vaucheret@versailles.inra.fr (H. Vaucheret).
[1] These two authors contributed equally to this work.

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interfering RNAs (siRNAs), the former deriving from single-stranded (ss)RNAs folded into short imperfect stem-loop structures, and the later from true dsRNAs, resulting from the folding of long inverted repeats (IR), convergent transcription or the action of RNA-dependent RNA polymerases (RDR) on ssRNAs. Later on, PIWI-related RNAs (piRNAs) were discovered, which are only found in animals whereas miRNAs and siRNAs are found in most eukaryotes. Unlike miRNAs and siRNAs, which are produced by DICER-type RNAseIII enzymes, piRNAs are produced in a DICER-independent manner through a ping-pong mechanism involving PIWI proteins [29]. miRNAs, siRNAs and piRNAs associate with Argonaute (AGO) proteins to guide TGS or PTGS on cognate targets based on their homology. More recently, the term scanning small RNA (scanRNA) has been coined [30] to refer to small RNAs that allow eliminating DNA in protists, while QDE-2-interacting small RNAs (qiRNAs) [26] and DSB-induced small RNAs (diRNAs) [28] refer to small RNAs that are induced at DNA double-stranded breaks (DSB) and participate in DNA repair in *Neurospora* and *Arabidopsis*, respectively [25–28].

3. Current models of the diverse plant endogenous small RNA pathways

The endogenous small RNA repertoire of wild-type plants grown under standard conditions consists of 10% miRNAs and 90% siRNAs [31], diRNAs being produced only when DSB are induced. Among the siRNA category, different types exist, including *trans*-acting siRNAs (ta-siRNA), natural antisense transcript-derived siRNAs (nat-siRNA), endogenous siRNAs (endo-siRNA), DNA-Dependent RNA Polymerase lV (PolllV)/PolV siRNAs (p4/p5-siRNA) and Needed for RDR2 Independent DNA Methylation (NERD) siRNAs.

3.1. MicroRNAs (miRNAs)

miRNAs derive from long single-stranded primary transcripts (pri-miRNA) that fold into stem-loop secondary structures (Fig. 1). pri-miRNAs are synthesized from specific non-protein-coding *MIR* genes by PolII. Similarly to protein coding transcripts, pri-miRNAs have typical PolII cap structures at their 5′ end and poly(A) tails at their 3′ end, and often contain introns [32]. Because of their intramolecular sequence complementarity, pri-miRNAs adopt a fold-back stem-loop structure and thus miRNA biogenesis does not require an RDR. The pri-miRNA is processed into mature miRNA by Dicer-like 1 (DCL1) in *Arabidopsis* [33–35]. Accurate maturation and processing of pri-miRNA requires the additional activity of several proteins, including the Cap-binding protein 20 (CBP20) and CBP80/ABH1 [36–38], the zinc finger protein Serrate (SE) [39,40], the dsRNA-binding protein/hyponastic leaves 1 (DRB1/HYL1) [41,42], the forkhead-associated (FHA) domain containing protein Dawdle (DDL) [43], the Tough protein (TGH) [44], the proline-rich protein Sickle (SIC) [45] and the RNA binding protein Modifier of SNC1 2 (MOS2) [46]. Whereas conserved, i.e. old, miRNAs are processed by DCL1 and DRB1 from short imperfectly paired stem-loops, non-conserved, i.e young, miRNAs are processed by DCL4 and DRB4 from long near-perfectly paired stem-loops [47,48], suggesting that *MIR* genes derive from long inverted repeats likely resulting from genomic duplications.

miRNAs are methylated at their 3′ terminal nucleotide by the RNA methyltransferase Hua Enhancer 1 (HEN1) [49–51] and most of them are exported to the cytoplasm by the exportin-5 homologue Hasty (HST) [52]. One strand of the miRNA duplex is subsequently incorporated into an RNA-induced silencing complex (RISC), which contains, at least, an AGO protein. Plant miRNAs promote the cleavage of their target RNA, to which they bind perfectly or near-perfectly, by employing mostly AGO1 as the RNA slicer. Therefore, cleavage is assumed as the common approach for miRNA-mediated gene regulation in plants [53–55]. However, in addition to regulating RNA degradation, miRNAs sometimes direct DNA methylation [56] or inhibit translation [57–62]. The rules governing the selection between the different modes of action are still not completely understood. Apart from the nature of the small RNA molecule, the identity of the AGO partner present in the RNA silencing complex deeply impacts the silencing outcome once engaged to the target. The identification of such partners and subsequent characterization of the associated small RNA molecules through specific AGO pull-down approaches uncovered distinct features for different AGO proteins [63–66]. Most miRNAs associate to AGO1. However, specific associations with AGO2 (miR408, miR393*), AGO7 (miR390) and AGO10 (miR165/166) have been reported. Although AGO1 per se is sufficient to promote RNA cleavage [53], in vivo AGO1 activity appears modulated, directly or indirectly, by several cellular effectors, including the plant orthologue of Cyclophilin 40 Squint (SQN), the Heat Shock Protein 90 (HSP90) [67], the F-Box protein FBW2 [68], the importin β protein enhanced miRNA activity (EMA1)/super sensitive to aba and drought 2 (SAD2) [69], the GW-proteins silencing defective 3 (SDE3) [70] and SUO [71]. Moreover the amount of AGO1 mRNA is regulated by AGO1 [62,72] and AGO10 [73]. AGO1 also associates with miR403 to regulate the amount of AGO2 mRNA [74], indicating complex interactions among AGO proteins.

3.2. Trans-acting siRNAs (ta-siRNAs)

ta-siRNAs derive from long non-coding transcripts of *trans acting siRNA* (*TAS*) genes that contain specific miRNA-binding sites (Fig. 1) [48,75–78]. Four types of *TAS* loci and three different miRNAs are involved in the biogenesis of ta-siRNAs. The *TAS* loci are transcribed into long non-coding RNAs by PolII and likely transferred by the THO/TREX complex to miRNA/AGO catalytic centers [79,80], where they are cleaved by one of the three miRNAs and thus trigger ta-siRNA production. The three miRNAs that guide *TAS* mRNA cleavage are miR173, miR390 and miR828. miR390 loaded on AGO7 has the peculiarity of triggering ta-siRNA production through double targeting of the *TAS3* transcript [64,81]. However, due to mismatches at the 5′ miR390-binding site, only the 3′ miR390-binding site is cleaved by the miR390/AGO7-containing complex [64]. On the other hand, miR173 [82], and most probably miR828, are loaded on AGO1 as the rest of miRNAs to guide cleavage of their RNA target. After RNA precursor cleavage, the RNA binding suppressor of gene silencing 3 (SGS3) protein stabilizes the cleavage products, which likely prevents their degradation, and allows recruiting RDR6 which, assisted by the putative RNA export factor SDE5, catalyzes the synthesis of a second complement RNA strand [78,79,83,84]. Next, DCL4 assisted by its interacting partner DRB4 processes the dsRNA to generate a population of 21-nt ta-siRNAs in phase with the miRNA guided cleavage site [85–87]. Thus, the initial cleavage site guided by the miRNA determines the ta-siRNAs sequence and subsequently its targets [48,75–78,81,82]. Similarly as miRNAs, ta-siRNAs duplexes are methylated by HEN1 [50] and one strand of the duplex associates with AGO1 to guide cleavage of target mRNAs [88]. Like most miRNAs, ta-siRNAs are involved in development. For example, the expression of *auxin response factor* (*ARF*) gene family members is regulated by a subset of ta-siRNAs, therefore controlling the vegetative phase transition in *Arabidopsis* [89–93].

3.3. Natural antisense transcripts-derived siRNAs (nat-siRNAs)

nat-siRNAs originate from dsRNA precursors resulting from the pairing of natural antisense transcripts (NAT). *cis*-NATs are transcribed from genes encoded by complementary strands of DNA at the same locus (Fig. 1), whereas *trans*-NATs are transcribed from two distinct genomic loci. In both cases, co-expression of overlapping sense/antisense transcripts could potentially form dsRNAs. *cis*-NATs usually have a long perfect complementary overlap between the sense and antisense transcripts, whereas the *trans*-NATs often have short and imperfect complementarity [94]. Despite the fact that dsRNAs could result from the annealing of sense/antisense transcripts, it has been