1 Diversity of RNA Silencing Pathways in Plants

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1.1 Introduction

RNA silencing is a manifestation of eukaryote defences against exogenous invading nucleic acids. Indeed, infection by pathogens, including fungi, bacteria, viruses or viroids, generally results in the production of pathogen-specific short interfering RNAs (siRNAs), the hallmark of RNA silencing (Hamilton and Baulcombe, 1999; Navarro *et al*., 2008). When loaded onto ARGONAUTE (AGO) proteins, these siRNAs guide the cleavage of the long RNAs naturally encoded by the invader (Vaucheret, 2008). However, despite the highly sequence-specific effect of siRNAs, pathogen-derived RNAs generally are not eliminated because most pathogens encode proteins that counteract the biogenesis or the action of siRNAs (Pumplin and Voinnet, 2013; Csorba *et al*., 2015).

RNA silencing is also used to control endogenous invading nucleic acids such as transposable elements (TE). In fact, TE silencing is mandatory to prevent uncontrolled expansion of these elements within the genome and avoiding subsequent deleterious effects, including gene disruption, gene activation or internal recombination. Unlike viruses, TEs generally do not encode proteins that have the capacity to block RNA silencing. Therefore, TEs generally are efficiently controlled by RNA silencing. Nevertheless, the protection of TE RNAs by TE proteins has been reported (Mari-Ordonez *et al*., 2013). Moreover, TE silencing can be erased under certain stress conditions (for example heat stress), leading to transient expression of TE RNAs and possible TE movement (Pecinka *et al*., 2010; Ito *et al*., 2011).

In contrast to pathogens and TEs, endogenous protein-coding genes generally are not a source for siRNA production and therefore are not subjected to RNA silencing. Indeed, only a handful of endogenous genes, in particular varieties, have been shown to produce siRNAs at levels that allow blocking transcription (transcriptional gene silencing or TGS) or degrading mRNAs (post-transcriptional gene silencing or PTGS), depending whether the siRNAs derive from the promoter or the transcribed region. Remarkably, these varieties exhibit genomic rearrangements, involving either duplication events or TEs inserted within or adjacent to the gene, whereas

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regular varieties that lack such rearrangements do not produce siRNAs and do not show silencing (Coen and Carpenter, 1988; Bender and Fink, 1995; Cubas *et al*., 1999; Clough *et al*., 2004; Tuteja *et al*., 2004; Della Vedova *et al*., 2005; Manning *et al*., 2006; Martin *et al*., 2009; Tuteja *et al*., 2009; Durand *et al*., 2012). It is assumed that genomic rearrangements resulting in the silencing of endogenous protein-coding genes are tolerated because they affect dispensable genes, and that cells undergoing genomic rearrangements that provoke the silencing of essential genes do not survive. This hypothesis implies that, during evolution, endogenous protein-coding genes are shaped to avoid producing siRNAs and undergoing silencing.

1.2 Transgene-based Genetic Screens to Unravel Silencing Pathways

The situation of endogenous protein-coding genes contrasts sharply to that of transgenes, which often undergo RNA silencing, although they are designed to structurally resemble and function like endogenous protein-coding genes. Note that RNA silencing was actually discovered as an unintended consequence of plant transformation (Matzke *et al*., 1989; Napoli *et al*., 1990; Smith *et al*., 1990; van der Krol *et al*., 1990). Indeed, it is now known that introduction of transgenes in the form of naked DNA, or by infection with disarmed bacteria such as *Agrobacterium*, always activates the production of siRNAs (Llave *et al*., 2002). Following stable integration in the genome, transgenes are either expressed or silenced. Nevertheless, silencing sometimes occurs after a period of normal expression that can last several generations. The reasons why certain transformants express a transgene whereas others undergo silencing by TGS or PTGS remain not well understood, and this raises important issues about the reliability of transgene expression. Importantly, when the transgene undergoing silencing carries sequences derived from an endogenous gene, transgene-derived siRNAs also affect the endogenous copy or copies, a phenomenon referred to as co-suppression (Napoli *et al*., 1990).

The fact that transgenes frequently undergo silencing whereas endogenous protein-coding genes do not, indicates that transgenes are often perceived as invaders that need to be silenced like pathogens or TEs. During the transient phase of extra-chromosomal expression, transgenes are generally present in high copy number, which may result in abnormally high levels of RNAs, thus mimicking what happens with invader RNAs during an infection, and activation of RNA silencing. Following integration in genomic areas allowing high levels of transcription, transgenes can still continue to produce high levels of RNAs, thus maintaining RNA silencing active against them. Supporting this hypothesis, transgenes that carry strongly expressed promoters are generally more prone to undergo silencing than transgenes that carry weakly expressed promoters. Stable integration of several transgene copies within the genome can also activate anti-transposons RNA silencing. Supporting this second hypothesis, transgenic plants exhibiting high transgene copy numbers are generally more prone to undergo silencing than plants carrying single copies.

Almost 20 years ago, the first forward genetic screens based on the reactivation of silenced transgenes identified the core components of the PTGS and TGS pathways. Enhancer screens were then set up, revealing cellular functions that antagonize silencing. More recently refined genetic screens, including sensitized

screens and suppressor screens, have allowed identification of a variety of regulatory components. So far, 12 and 18 forward genetic screens dedicated to PTGS and TGS, respectively, have been published. The outcome of these screens is described in Table 1.1 and Table 1.2. Because transgenes only serve as excellent reporters of endogenous functions, we do not describe further how each transgene locus is silenced. In the next sections, we describe what transgene-based genetic screens have told us about natural silencing pathways.

1.3 PTGS Pathways

1.3.1 Antiviral PTGS

Antiviral PTGS starts by the processing of virus-derived dsRNA into 21- and 22-nt primary siRNAs by DICER-LIKE 4 (DCL4) and DCL2, respectively (Bouche *et al*., 2006; Deleris *et al*., 2006; Fusaro *et al*., 2006). Virus-derived dsRNA molecules represent either: (i) the natural form of dsRNA viruses; (ii) intermediate forms of the replication of ssRNA viruses; (iii) partially folded viral ssRNAs; or (iv) molecules resulting from the action of RNA-DEPENDENT-RNA-POLYMERASE (RDR) enzymes on aberrant or subgenomic viral ssRNA. Primary siRNAs are methylated at their 3´ end by the methyltransferase HUA ENHANCER 1 (HEN1) (Boutet *et al*., 2003; Li *et al*., 2005) before loading onto AGO proteins, mainly AGO1 and AGO2 but also AGO5 or AGO7 (Morel *et al*., 2002; Qu *et al*., 2008; Harvey *et al*., 2011; Wang *et al*., 2011b; Brosseau and Moffett, 2015) to guide the cleavage of viral ssRNA through sequence homology. AGO-mediated cleavage generates RNA fragments that escape degradation due to the protective activity of SUPPRESSOR OF GENE SILENCING 3 (SGS3) (Mourrain *et al*., 2000; Yoshikawa *et al*., 2013). With the assistance of the putative RNA export protein SILENCING-DEFECTIVE (SDE5) (Hernandez-Pinzon *et al*., 2007), SGS3-protected cleavage products are transformed into dsRNA by RDR6 (Mourrain *et al*., 2000). These dsRNA are processed into siRNA duplexes by DCL4 to produce secondary siRNAs that reinforce AGO-mediated RNA cleavage, thus creating an amplification loop (Fig. 1.1). Such a process should eliminate viral RNA; however, most viruses have developed strategies to handle PTGS by expressing proteins called VSR (viral suppressors of RNA silencing), which block one or other of the steps of the PTGS pathway (Pumplin and Voinnet, 2013; Csorba *et al*., 2015).

This antiviral PTGS model also explains how PTGS is activated against sense transgenes that are not supposed to produce dsRNAs. Accordingly, transgenes that produce aberrant RNAs in sufficient amounts to escape degradation by nuclear and cytoplasmic RNA quality control (RQC) pathways (see below) are transformed into dsRNA by RDR6. The nature of transgene aberrant RNAs has long remained a mystery until the recent identification of uncapped transgene RNAs resulting from the 3´ end processing of readthrough transcripts (Parent *et al*., 2015b). RDR6-derived transgene dsRNAs are processed into 21-nt and 22-nt primary by DCL4 and DCL2 (Parent *et al*., 2015a), and loaded onto AGO1, which cleaves complementary target RNAs (Morel *et al*., 2002; Baumberger and Baulcombe, 2005). Transgene RNA cleavage fragments are transformed into

Table 1.1. Mutants identified in PTGS genetic screens.

Reporter gene/ transgene	Basis of the screen	Mutants nomenclature	Mutants	Gene product
p35S:rolB	Wild-type phenotype	*enhancer of gene silencing (egs)*	*egs1*	Unknown
			egs2	Unknown
p35S:GUS (*L1*)	GUS expression	*supressor of gene silencing (sgs)*	*sgs1*	Transcription factor NAC52
			sgs2	RNA dependent RNA polymerase RDR6
			sgs3	RNA binding protein SGS3
			sgs4	RNA slicer AGO1
			sgs5	RNA methylase HEN1
			sgs6	DNA methyltransferase MET1
			sgs7	RNA export protein SDE5
			sgs8	H3K4me2/3 demethylase JMJ14
			sgs9	RNA traficking protein HPR1
p35S:NIA2 (*2a3*)	Wild-type growth	*supressor of gene silencing (sgs)*	*sgs2*	RNA dependent RNA polymerase RDR6
			sgs3	RNA binding protein SGS3
			sgs13	RNA helicase protein SDE3
			sgs14	Small nuclear protein SmD1
			sgs17	Cyp40 like gene SQUINT
p35S:GUS (L1) in *ago1-27* or *ago1-33*	Absence of GUS activity	none	*fry1*	Inositol polyphosphoric acid 1-phosphatase FRY1
			ski3	Exosome cofactor SUPERKILLER3
p35S:MP17-GFP	Wild-type phenotype	*increased transgene silencing (its)*	*xrn4*	5´-3´ exoribonuclease XRN4
			dcp2	mRNA decapping enzyme DCP2
p35S:GFP x *p35S: PVX-GFP* (*GxA*)	GFP expression	*silencing defective (sde)*	*sde1*	RNA-dependent RNA polymerase RDR6
			sde2	RNA binding protein SGS3
			sde3	RNA helicase protein SDE3
			sde4	PolIV subunit NRPD1
			sde5	RNA export protein SDE5
			sde6	RNA-dependent RNA polymerase RDR2
p35S:PVX-GFP in *rdr6*	enhanced GFP expression		*dcl4*	DICER-LIKE protein DCL4
			hen1	RNA methylase HEN1
			upf1	NMD factor UPFRAMESHIFT1

p35S:PVX-PDS	Increase in *PDS* silencing	*enhanced silencing phenotype (esp)*	*esp1*	mRNA 3´ formation CstF64-like
			esp3	RNA helicase splicing factor PRP2
			esp4	mRNA 3´ formation symplekin
			esp5	mRNA 3´ formation CPSF100
p35S:GFP-miR171 (*GFP171.1*)	GFP expression	*microrna biogenesis deficient (mbd)*	*mbd1*	DICER-LIKE protein DCL1
			mbd2	RNA methylase HEN1
		microrna action deficient (mad)	*mad3*	HMGR enzyme
			mad4	Sterol C-8 isomerase
			mad5	Microtubule severing protein KTN1
			mad7	Exosome cofactor SUPERKILLER2
p35S:LUC and *p35S:miR-LUC*	LUC expression	none	*cpl1*	C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1
p35S:TAS3aPDS (*TAS3-syn*)	Decreased photobleaching	none	*ago7*	RNA slicer AGO7
			dcl4	DICER-LIKE protein DCL4
			rdr6	RNA-dependent RNA polymerase RDR6
			sgs3	RNA binding protein SGS3
			mir390	*MIR390*
pSUC2:SUL	Decreased photobleaching	*silencing movement-deficient (smd)*	*smd1*	RNA dependent RNA polymerase RDR2
			smd2	PolIV largest subunit NRPD1
			dcl4	DICER-LIKE protein DCL4
pSUC2:PDS (*JAP3*)	Decreased photobleaching	none	*clsy1*	SNF2 domain–containing protein CLSY1
			nrpd1	Nuclear RNA polymerase D1
			rdr2	RNA-dependent RNA polymerase RDR2
			dcl3	DICER-LIKE protein DCL3
			nrpd2	Nuclear RNA polymerase D2
			fca	mRNA processing protein FCA
			fpa	mRNA processing protein FPA
			jmj14	H3K4me2/3 demethylase JMJ14
			tex1	RNA trafficking protein TEX1

Table 1.2. Mutants identified in TGS genetic screens.

Reporter gene/transgene	Basis of the screen	Mutant nomenclature	Mutants	Gene product
p35S:CHS-pNos:NptII (C)	Decreased anthocyanin	*homology dependent gene silencing (hog)*	*hog1*	S-Adenosyl-homocysteine hydrolase SAHH1
	Kanamycin resistance	*modifier of silencing (sil)*	*sil1*	Rpd3-like histone deacetylase HDA6
p35S:HPT (A)	Hygromycin resistance	*somniferous effect (som)*	*som1*	Chromatin remodelling factor DDM1
			mom1	Chromatin remodelling factor MOM1
pUBQ3:LUC in mom1	LUC expression	*mom1 enhancer (moe)*	*moe1*	PolV subunit NRPE1
SUP in clk (clk-st)	Wild-type flowers	*none*	*cmt3*	Cytosine methyltransferase CMT3
			kyp	H3K9 methyltransferase SUVH4
			ago4	RNA slicer AGO4
Endogenous PAI1-PAI4 in Ws	Reduced UV fluorescence	*none*	*cmt3*	Cytosine methyl transferase CMT3
			suvh4	H3K9 methyltransferase SUVH4
p35S::GUS (L5)	GUS expression	*none*	*rpa2*	Replication protein A 2nd subunit RPA2
pRD29A:LUC	Impaired LUC expression	*repressor of silencing (ros)*	*ros1*	Glycosylase ROS1
			ros3	Regulator of DNA demethylation ROS3
			ros4	Acetyltransferase protein IDM1
pRD29A:LUC in ros1	LUC expression	*rna-directed dna methylation (rdm)*	*rdm1*	RNA-directed DNA methylation RDM1
			rdm2	PolIV/PolV subunits NRPD4/NRPE4
			rdm3	KOW domain-containing transcription factor KTF1
			rdm4	Transcription factor for both PolII and PolIV
			rdm5	PolIV subunit NRPD1
			rdm6	PolV subunit NRPE1
			rdm7	PolIV subunit NRPD2a
			rdm8	RNA slicer AGO4
			rdm9	RNA methylase HEN1
			rdm10	Chromatin remodelling factor DRD1

			rdm11	Rpd3-like histone deacetylase HDA6
			rdm12	Similarities with SGS3/ partner for RDR2 RDM12/IDN2
			rdm16	Splicing factor PRP3
			rpa2	Replication protein A 2nd subunit RPA2
			ago6	RNA slicer AGO6
			ubp26	Ubiquitin protease UBP26
			dtf1	Putative DNA binding transcription factor DTF1
			zop1	Zinc finger (ZnF) and OCRE domain-containing protein ZOP1
			dtf1	Sawadee Homeodomain Homolog protein SHH1
			prp31	Splicing factor PRP31
Ectopic FWA copies	Late flowering phenotype	*involved in de novo DNA methylation (idn)*	*idn1*	Similar to the hinge-domain region of structural maintenance of chromosomes
			idn2	SGS3-like partner for RDR2 RDM12/ IDN2
			sr45	Splicing factor Serine-arginine rich SR45
			drm2	Domains rearranged methyltransferase DRM2
			dcl3	DICER-LIKE protein DCL3
			rdr2	RNA dependent RNA polymerase RDR2
			ago4	RNA slicer AGO4
			ago6	RNA slicer AGO6
			nrpe1	PolV subunit NRPE1
			drd1	Putative chromatin remodelling factor DRD1
			ktf1	KOW domain-containing transcription factor KTF1

Continued

Table 1.2. Continued.

Reporter gene/transgene	Basis of the screen	Mutant nomenclature	Mutants	Gene product
			clsy1	SNF2 domain-containing protein CLSY1
			nrpd/e2	PolIV/PolV subunit NRPD2/NRPE2
p35S:NOS x pNOS:NPTII (KxH)	Kanamycin resistance	*rna-mediated transcriptional silencing (rts)*	*rts1*	Rpd3-like histone deacetylase HDA6
			rts2	DNA methyltransferase MET1
p35S:α x pα:GFP	GFP expression	*defective in rna-directed dna methylation (drd)*	*drd1*	Chromatin remodelling factor DRD1
			drd2	PolIV/PolV subunit NRPD2/NRPE2
			drd3	PolV subunit NRPE1
p35S:ENH x pENH:GFP (SxT)	GFP expression	*defective in meristem silencing (dms)*	*dms1*	Putative chromatin remodelling factor DRD1
			dms2	PolIV subunit NRPD2a
			dms3	Similar to the hinge-domain region of structural maintenance of chromosomes
			dms4	Putative transcription factor IWR1
			dms5	PolIV subunit NRPD1b
			dms6	DICER-LIKE protein DCL3
			dms7	RNA-directed DNA methylation RDM1
			dms8	Domains rearranged methyltransferase DRM2
			dms9	RNA slicer AGO6
			dms10	SGS3-like partner for RDR2 RDM12/IDN2
			dms11	ATPase DMS11/GHKL
pNOS:35S x p35S:GFP	GFP expression	*rna-directed dna methylation defective (rmd)*	*rmd1*	PolIV subunit NRPD1b

			rmd3	PolIV subunit NRPD1a
pSDC:GFP	GFP expression	*none*	*morc1*	ATPase-containing Microrchidia1 (MORC1)
			morc6	ATPase-containing Microrchidia1 (MORC6)
p35S:LUC-AP2 in rdr6 (LUCH)	Increase in LUC expression	*none*	*ago4*	RNA slicer AGO4
			drd1	Chromatin remodelling factor DRD1
			drm2	Domains rearranged methyltransferase DRM2
			mom1	Chromatin remodelling factor MOM1
			hen1	RNA methylase HEN1
			amp1	ER-associated ALTERED MERISTEM PROGRAM1
			suvh1	H3K4 methyltransferase SUVH1
p35S:LUC-AP2 in rdr6 (LUCL)	Increase in LUC expression	*none*	*top1a*	DNA topoisomerase 1a
p35S:SUC2	Restored root growth	*asi (anti-silencing)*	*asi1*	RNA binding protein IMB2
			ros1	Glycosylase ROS1
			ros4	Acetyltransferase protein IDM1
pAPUM9:GFP (Silex)	Restored GFP expression	*epic (epigenetic control)*	*hda6*	Rpd3-like histone deacetylase HDA6

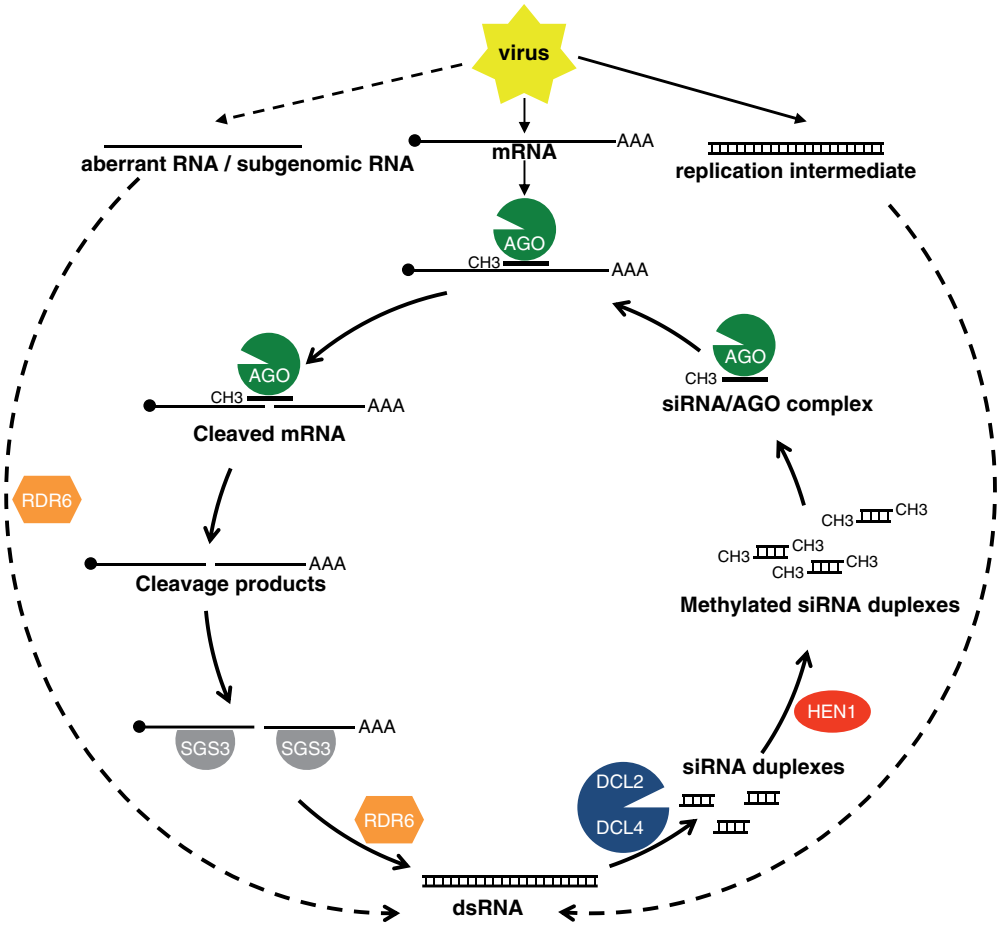

Fig. 1.1. Model for antiviral PTGS. Dashed arrows indicate putative initiation routes. Plain arrows indicate the amplification step. See section 1.3.1 of the text for details on the mechanisms and for additional actors involved.

dsRNA through the action of SGS3, SDE5 and RDR6 (Mourrain *et al.*, 2000; Jauvion *et al.*, 2010) and processed into siRNA duplexes by DCL4 to produce secondary 21-nt siRNAs that reinforce the cleavage of transgene mRNA through AGO1. Additional factors contribute to the efficiency of transgene PTGS, for example the RNA helicase SDE3 that binds to AGO1 (Dalmay *et al.*, 2001; Garcia *et al.*, 2012), or the RNA trafficking protein HYPER RECOMBINATION 1 (HPR1), which is likely to play a role in bringing RNA molecules to the right place during PTGS (Hernandez-Pinzon *et al.*, 2007; Jauvion *et al.*, 2010; Yelina *et al.*, 2010). In addition, the nuclear ribonucleoprotein SmD1 is likely to facilitate PTGS by protecting transgene aberrant RNAs from degradation by the RQC machinery in the nucleus, thus increasing the amount of transgene aberrant RNAs that succeed in entering siRNA-bodies in the cytoplasm to eventually activate PTGS (Elvira-Matelot *et al.*, 2016b).

1.3.2 RQC as a first layer of defence limiting PTGS

RQC encompasses RNA decay pathways that ensure the elimination of error-bearing RNAs. RQC should therefore eliminate the aberrant RNAs that activate PTGS. However, PTGS is regularly activated against pathogens and transgenes, probably because the amount of aberrant RNAs produced by viruses and transgenes exceeds the capacity of RQC pathways.

RQC generally involves the removal of the 5′ cap and/or the 3′ poly(A) tail. The removal of either modification is initiated when RNAs are not properly processed or translated. For example, when translation is arrested either owing to the presence of a premature termination codon or owing to excessive 3′ untranslated region (UTR) length, a process referred to as nonsense-mediated decay (NMD) is activated (Belostotsky and Sieburth, 2009). NMD generally involves the recruitment and activation of conserved UPFRAMESHIFT 1 (UPF1), UPF2 and UPF3 proteins to defective transcripts that are translationally stalled. This recruitment, either by invoking decapping and deadenylation pathways or via endonucleolytic cleavage, generates aberrant RNAs that are subsequently degraded through exonucleolytic cleavage. In *Arabidopsis*, the removal of the cap structure is catalysed by a set of conserved proteins that constitute the decapping complex, including DECAPPING1 (DCP1), DCP2, DCP5, VARICOSE (VCS) and DEAD BOX HELICASE HOMOLOG1 (DHH1) (Xu *et al*., 2006; Goeres *et al*., 2007; Iwasaki *et al*., 2007). On the other hand, the shortening of the 3′ poly(A) tail (deadenylation) is catalysed by the conserved 3′-to-5′ POLY(A)-SPECIFIC RIBONUCLEASE (PARN) as well as by the conserved CARBON CATABOLITE REPRESSOR 4 (CCR4) complex (Belostotsky and Sieburth, 2009). The 5′-to-3′ XRN exoribonucleases degrade RNA with unprotected 5′ ends (Kastenmayer and Green, 2000), whereas the multimeric exosome complex contains 3′-to-5′ exoribonucleases that degrade RNA with unprotected 3′ ends (Chekanova *et al*., 2007). *Arabidopsis* expresses three XRN proteins, the nucleolar XRN2, the nucleoplasmic XRN3, and cytoplasmic XRN4 (Kastenmayer and Green, 2000). Biochemical and molecular characterization of the *Arabidopsis* exosome core complex identified nine subunits: RIBOSOMAL RNA PROCESSING4 (RRP4), RRP40, RRP41, RRP42, RRP43, RRP45, RRP46, CENTROMERE ENHANCER OF POSITION EFFECT1 SYNTHETIC LETHAL PROTEIN4 (CLS4) and mRNA TRANSPORT REGULATOR3 (MTR3) (Chekanova *et al*., 2007), plus specific cofactors that confer subcellular specialization; for example, MTR4 in the nucleolus, HEN2 in the nucleoplasm and the SUPERKILLER (SKI) complex in the cytoplasm (Lange *et al*., 2014; Yu *et al*., 2015; Zhang *et al*., 2015).

Whereas RQC and PTGS were originally considered as exclusive pathways, eliminating endogenous aberrant RNAs and exogenous RNAs, respectively, it turned out that RQC generally serves as a first layer of defence against aberrant RNAs of both origins, and that PTGS is activated when RQC is unable to eliminate these aberrant RNAs. Indeed, compromising NMD factors UPF1 or UPF3, decapping enzymes DCP1, DCP2 or VCS, 5′-to-3′ exoribonucleases XRN2, XRN3 or XRN4, exosome core subunits RRP4 or RRP41, or exosome cofactors RRP6L1, MTR4, HEN2 or SKI3 enhance transgene PTGS (Gazzani *et al*., 2004; Gy *et al*., 2007; Thran *et al*., 2012; Moreno *et al*., 2013; Lange *et al*., 2014; Zhang *et al*., 2015; Hematy *et al*., 2016), indicating that RQC limits the efficiency of PTGS. Moreover, mutations in XRN4 or

UPF1 also affected the efficiency of antiviral PTGS (Gy *et al*., 2007; Garcia *et al*., 2014). It is likely that aberrant RNAs are first exposed to degradation by RQC, and only if RQC is compromised or saturated do aberrant RNAs enter into siRNA-bodies where they are transformed into double-stranded RNA (dsRNA) by cellular RDR, thus allowing the production of siRNAs and the sequence-specific degradation of both functional and dysfunctional homologous mRNAs. Supporting this hypothesis, transgene loci that spontaneously trigger PTGS were found to produce uncapped RNAs at much higher levels than transgene loci that do not spontaneously trigger PTGS (Parent *et al*., 2015b). Moreover, mutating XRN4 results in increased levels of uncapped RNAs from non-spontaneously triggering loci and subsequent triggering of PTGS (Parent *et al*., 2015b). Also supporting the hypothesis that PTGS is triggered when RQC capacity is exceeded, P-bodies (where decapping enzymes reside) and siRNA-bodies (where cellular RDR6 resides) were found to constitute two distinct but adjacent foci (Jouannet *et al*., 2012; Moreno *et al*., 2013; Martínez de Alba *et al*., 2015), suggesting that after saturating the degradation capacity of P-bodies, aberrant RNAs can move to siRNA-bodies to activate PTGS.

Remarkably, compromising decapping in *dcp2* and *vcs* mutants, or compromising both 5´-to-3´ and 3´-to-5´ RNA degradation in the *xrn4 ski2* double mutant provokes the entry of hundreds of endogenous mRNAs into the PTGS pathway and the production of siRNAs referred to as RNA quality control-specific siRNAs (rqc-siRNAs) or coding transcripts siRNAs (ct-siRNAs), respectively (Martínez de Alba *et al*., 2015; Zhang *et al*., 2015). In the conditions tested, ~1800 endogenous mRNAs produce rqc-siRNAs (Martínez de Alba *et al*., 2015), while ~450 endogenous mRNAs produce ct-siRNAs (Zhang *et al*., 2015), among which ~200 are common. Most of the ct-siRNAs identified in the *xrn4 ski2* double mutant depend on RDR6 for their production (441 out of 456), whereas only part of the rqc-siRNAs identified in *dcp2* and *vcs* mutants depend on RDR6 (350 out of 1785). Since rqc-siRNAs come from both strands, it is likely that another cellular RDR is at play for the production of certain rqc-siRNAs. RDR1 is a good candidate. Indeed, RDR1 has been recently implicated in the production of another category of endogenous siRNAs, called virus-activated siRNAs (vasiRNAs), which are produced from ~1200 endogenous protein-coding genes when plants are infected by viruses (Cao *et al*., 2014). Most of the vasiRNAs identified in virus-infected plants depend on RDR1 for their production (1068 out of 1172). Remarkably, ~350 genes producing vasiRNAs in virus-infected plants produce rqc-siRNAs in *dcp2* and *vcs* mutants, supporting the hypothesis that RDR1 participates in the production of rqc-siRNAs in decapping mutants. These results also suggest that viruses could provoke the production of siRNAs from endogenous protein-coding genes by inhibiting RQC mechanisms, or by stimulating the production of aberrant RNAs up to a level that saturates the RQC pathway and triggers their entry into the PTGS pathway.

1.3.3 Specialized PTGS pathways directed against certain endogenous mRNA

As shown above, endogenous mRNAs are usually not targeted by PTGS because RQC pathways have evolved to efficiently eliminate aberrant RNAs produced by endogenous genes without producing siRNAs that could destroy functional mRNAs.

Nevertheless, plants and other eukaryotes have evolved specialized PTGS pathways to selectively regulate the abundance of certain endogenous mRNAs through the action of particular small RNAs, namely microRNAs (miRNAs), trans-acting siRNAs (ta-siRNAs) and natural antisense siRNAs (nat-siRNAs) (Fig. 1.2).

MIR genes are transcribed by PolII into long single-stranded primary transcripts (pri-miRNA), which exhibit typical PolII cap structures at their 5´ end and poly(A) tails at their 3´ end, and often contain introns (Jones-Rhoades *et al*., 2006). They adopt a fold-back stem-loop structure that is processed into a mature miRNA duplex by DCL1 in *Arabidopsis* (Park *et al*., 2002; Reinhart *et al*., 2002; Kurihara and Watanabe, 2004). Accurate maturation and processing of pri-miRNA also requires the Cap-binding protein 20 (CBP20) and CBP80/ABH1 (Gregory *et al*., 2008; Kim *et al*., 2008; Laubinger *et al*., 2008), the zinc finger protein SERRATE (SE) (Lobbes *et al*., 2006; Yang *et al*., 2006), the dsRNA binding protein/HYPONASTIC LEAVES 1 (DRB1/HYL1) (Han *et al*., 2004; Vazquez *et al*., 2004a), the Forkhead-associated (FHA) domain-containing protein DAWDLE (DDL) (Yu *et al*., 2008), the TOUGH protein (TGH) (Ren *et al*., 2012), the Proline-rich protein SICKLE (SIC) (Zhan *et al*., 2012) and the RNA-binding protein MODIFIER OF SNC1, 2 (MOS2) (Wu *et al*., 2013). miRNAs are methylated at their 3´ terminal nucleotide by the RNA methyltransferase HEN1 (Boutet *et al*., 2003; Li *et al*., 2005; Yu *et al*., 2005) and most are exported to the cytoplasm by the exportin-5 homologue HASTY (HST) (Park *et al*., 2005). One strand of the miRNA duplex acts as a guide strand and is selectively loaded onto an AGO protein, whereas the other strand, the passenger strand (miRNA*) is discarded from the complex and rapidly degraded. Most miRNAs associate to AGO1. However, specific association of miR408 or miR393* with AGO2, of miR390 with AGO7 and of miR165/166 with AGO10 have been reported (Mi *et al*., 2008; Montgomery *et al*., 2008a; Takeda *et al*., 2008; Zhu *et al*., 2011). 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 (Rhoades *et al*., 2002; Baumberger and Baulcombe, 2005; Schwab *et al*., 2005). However, in addition to regulating RNA degradation, miRNAs sometimes direct DNA methylation (Bao *et al*., 2004) or inhibit translation (Aukerman and Sakai, 2003; Chen, 2004; Gandikota *et al*., 2007; Brodersen *et al*., 2008; Lanet *et al*., 2009; Mallory *et al*., 2009). Although AGO1 per se is sufficient to promote RNA cleavage (Baumberger and Baulcombe, 2005), *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) (Smith *et al*., 2009), the F-Box protein FBW2 (Earley *et al*., 2010), the importin b protein ENHANCED miRNA ACTIVITY (EMA1)//SUPER SENSITIVE TO ABA AND DROUGHT 2 (SAD2) (Wang *et al*., 2011a), the GW-proteins SILENCING DEFECTIVE 3 (SDE3) (Garcia *et al*., 2012) and SUO (Yang *et al*., 2012). Moreover the amount of AGO1 mRNA is regulated by AGO1 (Vaucheret *et al*., 2004; Mallory and Vaucheret, 2009) and AGO10 (Mallory *et al*., 2009).

TRANS ACTING siRNA (*TAS*) genes are transcribed by PolII into long single-stranded RNAs that contain specific miRNA binding sites (Vazquez *et al*., 2004b; Allen *et al*., 2005; Vaucheret, 2005; Yoshikawa *et al*., 2005; Rajagopalan *et al*., 2006).

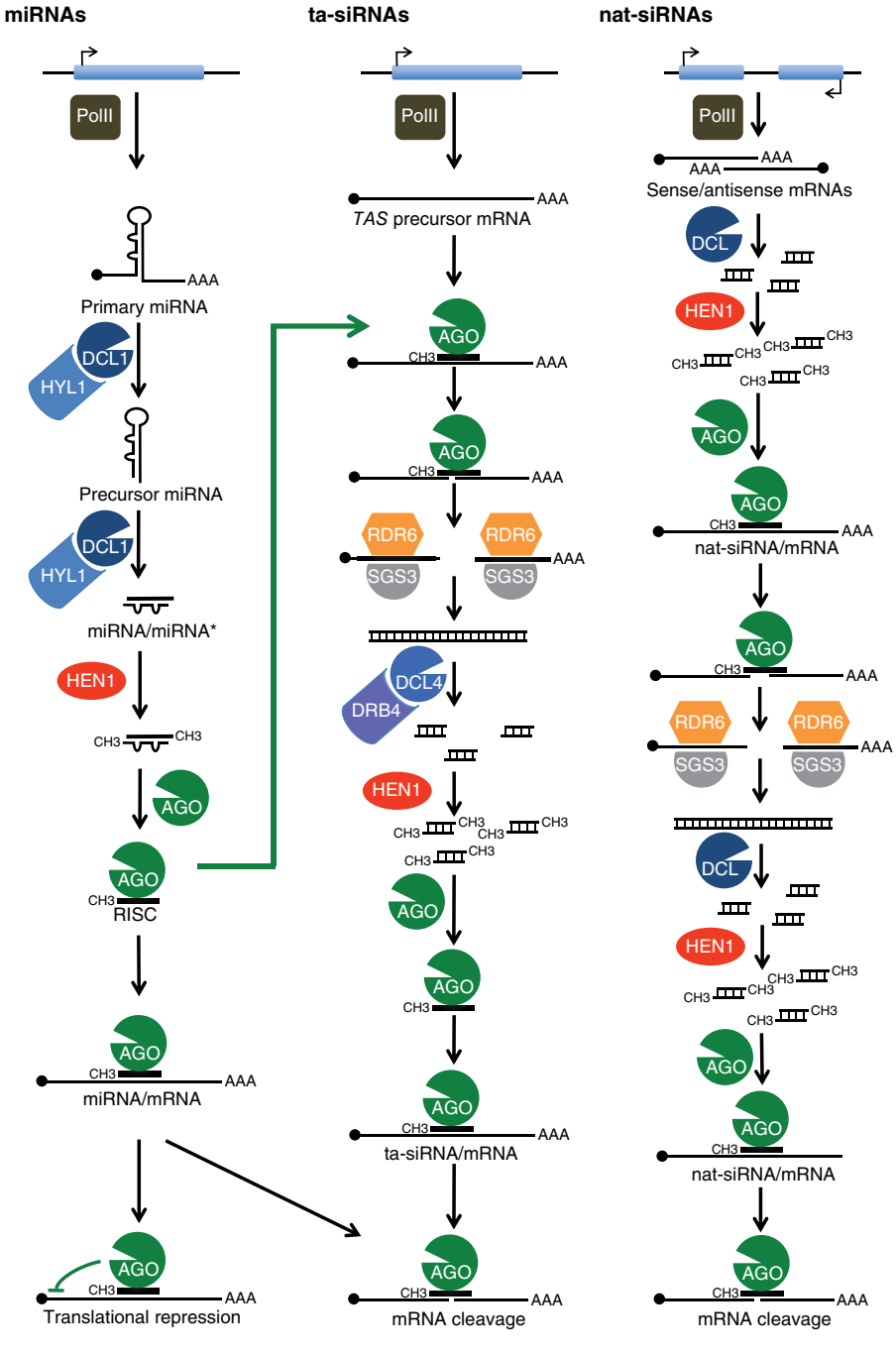

Fig. 1.2. Endogenous miRNA, ta-siRNA and nat-siRNA pathways. See section 1.3.3 of the text for details on the mechanisms and for additional actors involved.

It is likely that *TAS* RNAs are transferred by the THO/TREX complex to miRNA/AGO catalytic centres (Jauvion *et al*., 2010; Yelina *et al*., 2010). After cleavage, the RNA-binding SGS3 protein stabilizes the cleavage products, which probably prevents their degradation, allowing recruiting RDR6 which, assisted by the putative RNA export factor SDE5, catalyses the synthesis of a second complementary RNA strand (Yoshikawa *et al*., 2005; Hernandez-Pinzon *et al*., 2007; Elmayan *et al*., 2009; Jauvion *et al*., 2010). Next, DCL4 assisted by its interacting partner DsRNA BINDING PROTEIN 4 (DRB4) processes the dsRNA to generate a population of 21-nt ta-siRNAs in phase with the miRNA guided cleavage site (Gasciolli *et al*., 2005; Xie *et al*., 2005; Nakazawa *et al*., 2007). Thus, the initial cleavage site guided by the miRNA determines the ta-siRNAs sequence and subsequently its targets (Vazquez *et al*., 2004b; Allen *et al*., 2005; Vaucheret, 2005; Yoshikawa *et al*., 2005; Axtell *et al*., 2006; Rajagopalan *et al*., 2006; Montgomery *et al*., 2008b). Similar to most miRNAs, ta-siRNAs duplexes are methylated by HEN1 (Li *et al*., 2005) and one strand of the duplex associates with AGO1 to guide cleavage of target mRNAs (Allen and Howell, 2010).

If they are co-expressed, genes that are transcribed from complementary DNA strands at the same locus produce overlapping sense/antisense transcripts. Despite the fact that dsRNAs can result from the annealing of sense/antisense transcripts, the production of siRNAs referred to as nat-siRNAs not only requires a DCL, but also the activity of PolIV, RDR6 and SGS3 (Borsani *et al*., 2005; Katiyar-Agarwal *et al*., 2006). Primary nat-siRNAs are loaded onto a yet unidentified AGO protein to direct the cleavage of the constitutively expressed complementary transcript. In a second step, the cleaved transcript is converted into dsRNA in a PolIV-, RDR6- and SGS3-dependent manner (Borsani *et al*., 2005). This RNA amplification step may extend beyond the overlapping region to form siRNAs outside the overlapping region. Further processing of the newly synthesized dsRNA in a DCL1-dependent fashion would generate 21-nt nat-siRNAs, which target the constitutive expressed transcripts (Borsani *et al*., 2005). The RNA methyltransferase mutant *hen1* reduces the level of nat-siRNAs accumulation (Katiyar-Agarwal *et al*., 2006), indicating that nat-siRNAs are methylated by HEN1 like other siRNAs. The involvement of so many factors in the biogenesis of nat-siRNAs implies that multiple layers of control exist and that the formation of the NAT pair may be necessary but not sufficient for the generation of nat-siRNAs. Recent genome-wide analyses showed the widespread existence of overlapping sense/antisense transcripts, which raises the possibility that nat-siRNAs could be major effectors of gene regulation. Although it is still unclear how many of these converging transcripts lead to RNA silencing, a fast and controlled production of nat-siRNAs could govern a plant-adaptive protection mechanism in response to either abiotic or biotic stress (Borsani *et al*., 2005; Katiyar-Agarwal *et al*., 2006).

1.3.4 PTGS pathways directed against transposons

Besides protein-coding genes, plant genomes contain many repeated sequences, including transposons, which need to be silenced to avoid inducing mutations

and chromosome instability if multiplying within the genome. The way transposons and repeats are maintained in a transcriptionally silent state has been well deciphered (see TGS section below). However, how transposon silencing is initiated against active transposons is only starting to be understood. In *met1* mutants, loss of DNA methylation allowed reactivation of an intact *ATCOPIA93* family representative, *EVD18*. Crossing out *met1* allowed following the fate of this element, revealing that transposon mRNAs are first targeted by the PTGS machinery (RDR6, DCL4) to produce 21-nt siRNAs. However, these siRNAs fail to guide cleavage of transposon mRNAs because EVD encodes a nucleocapside that protects EVD mRNAs. Multiplication of the transposon leads to saturation of DCL4 and subsequent production of 24-nt by DCL3. These 24-nt siRNAs guide DNA methylation through AGO4, first within the EVD transcribed sequences, then spreading into the LTR (promoter) region, leading to TGS initiation (Mari-Ordonez *et al*., 2013).

An alternative pathway was revealed when looking at the fate of an Athila6A element reactivated in *ddm1* mutants. Indeed, 21- and 22-nt siRNAs resulting from the degradation of Athila6A mRNAs by the PTGS machinery (RDR6, DCL2, DCL4, AGO1) can be directly incorporated into AGO6 to guide DNA methylation (McCue *et al*., 2012; Nuthikattu *et al*., 2013; McCue *et al*., 2015) (Fig. 1.3).

Moreover, specific genomic loci, including TEs, were shown to undergo DNA methylation through atypical 21-22-nt siRNAs (Pontier *et al*., 2012). This alternative TGS pathway is independent of RdDM components (RDR2, AGO4), but depends on classical PTGS pathway components, such as RDR6 and AGO2 (Pontier *et al*., 2012). Moreover, this 21-22-nt-mediated DNA methylation pathway requires NEEDED FOR RDR2 INDEPENDENT DNA METHYLATION (NERD), a member of the GW repeat protein family, which generally binds to AGO proteins. NERD is thought to bind unmethylated histone H3 lysine 9 at specific genomic target loci and direct DNA methylation via its interaction with AGO2 bound to 21-22-nt siRNAs (Pontier *et al*., 2012) (Fig. 1.3).

At last, transposon mRNA were shown to be targeted by PTGS-derived 21-nt siRNAs in cells where DDM1 is naturally not expressed, i.e. in the vegetative nucleus of pollen grains and in dedifferentiated plant cell cultures. Indeed, 21-nt siRNAs – referred to as epigenetically activated small interfering RNAs – (easiRNAs) are produced by thousands of transposon transcripts that are specifically targeted by more than 50 miRNAs. Similar to ta-siRNAs, easiRNAs result from the transformation of cleavage products into dsRNA by RDR6 and processing by DCL4. Therefore, miRNA-directed easiRNA production appears as a mechanism that specifically targets transposons when they are epigenetically reactivated during reprogramming of the germ line (Creasey *et al*., 2014).

1.4 TGS Pathways

1.4.1 PolIV-RdDM pathway

Maintenance of transcriptional silencing at transposons and repeats involves PolIV-RdDM, i.e. RNA-directed DNA methylation mediated by PolIV-dependent

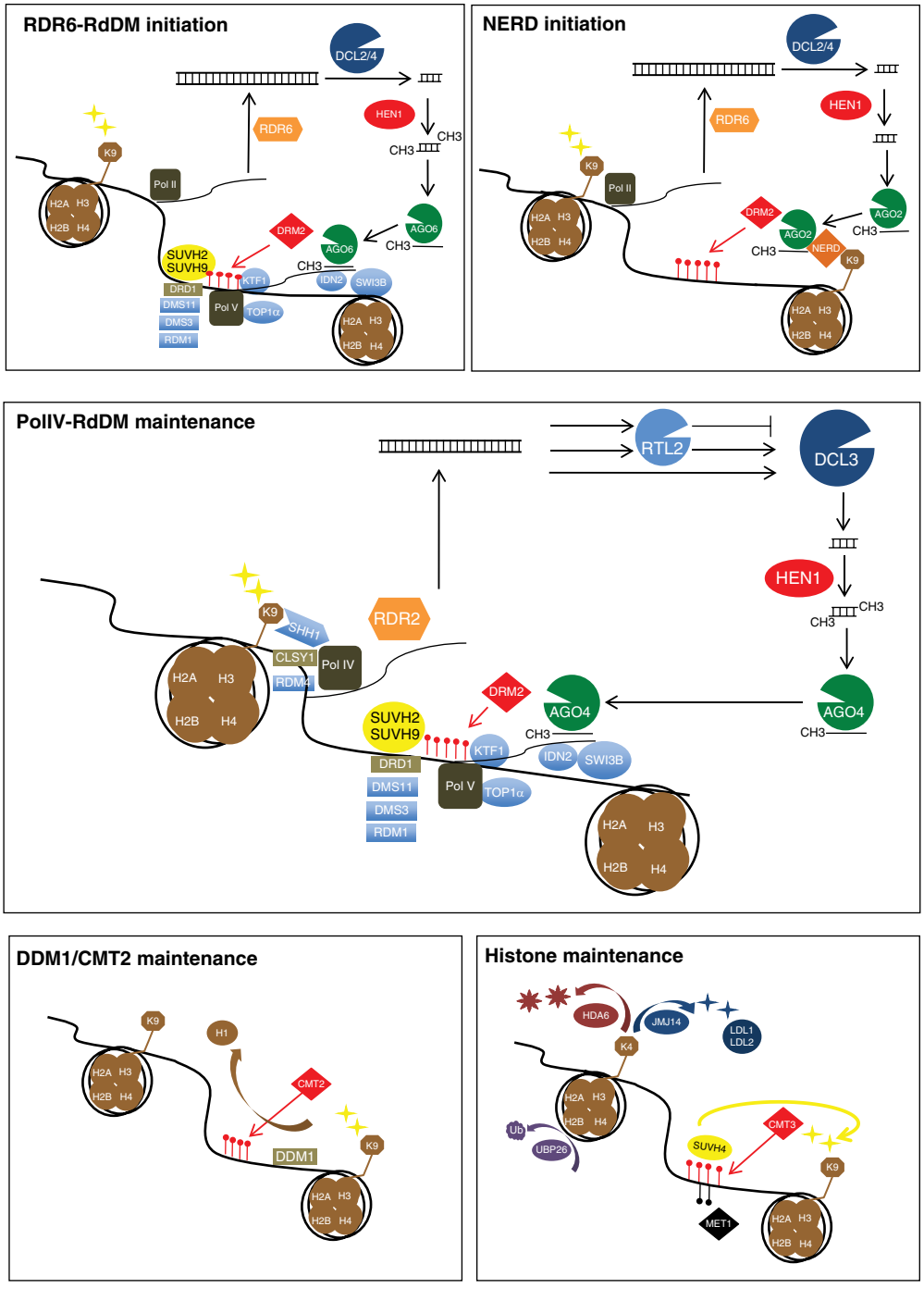

Fig. 1.3. Initiation and maintenance silencing pathways controlling transposons. See sections 1.3.4, 1.4.1 and 1.4.2 of the text for details on the mechanisms and for additional actors involved.

24-nt siRNAs (Henderson and Jacobsen, 2007; Zaratiegui *et al*., 2007). The biogenesis of most 24-nt siRNAs depends on the plant-specific DNA-dependent RNA polymerase PolIV, a derivative of PolII, which shares several subunits with PolII but also exhibits specialized subunits, including NRPD1. PolIV is assisted by CLASSY1 (CLSY1), a SNF2-like chromatin remodelling factor (Herr *et al*., 2005; Kanno *et al*., 2005; Onodera *et al*., 2005; Pontier *et al*., 2005) and SAWADEE HOMEODOMAIN HOMOLOG1 (SHH1), a homeodomain protein which recognizes H3K9me2 (He *et al*., 2009a; Law *et al*., 2011; Johnson *et al*., 2014). Single-stranded transcripts, referred to as P4-RNAs, are short, usually 27- to 45-nt in length (Blevins *et al*., 2015; Zhai *et al*., 2015). These precursors are depleted in both *polIV* and *rdr2* mutants, indicating that they are dependent on RDR2, reinforcing the idea that PolIV and RDR2 activities are coupled to produce P4R2 RNAs (Law *et al*., 2011; Haag *et al*., 2012). P4R2 RNAs are subsequently transformed into dsRNA through the action of RDR2 (Xie *et al*., 2004), in partnership with INVOLVED IN DE NOVO 2 (IDN2)/RNA DIRECTED DNA METHYLATION 12 (RDM12), an RNA binding protein similar to SGS3 (Zheng *et al*., 2010). Because of their short size, P4R2-RNAs each give rise to only one 24-nt siRNA, originating from either the 5′ or the 3′ end. These results fit well with the fact that DCL3 preferentially cleaves short precursors (Nagano *et al*., 2014). Before being processed by DCL3, a fraction of P4R2 RNAs is cleaved by RNASE THREE LIKE 2 (RTL2). Depending on the P4R2 RNA considered, RTL2-mediated cleavage either reduces or enhances the production of 24-nt siRNAs, indicating that RTL2 acts as a modulator of 24-nt siRNA production at defined loci (Elvira-Matelot *et al*., 2016a) (Fig. 1.3).

Like other small RNAs, DCL3-derived 24-nt siRNAs duplexes are methylated by HEN1 (Xie *et al*., 2004). One strand of the duplex is loaded into a RISC-like complex containing AGO4, AGO6 or AGO9 (Havecker *et al*., 2010). The complex formed by AGO proteins and 24-nt siRNA interacts with PolV-derived scaffold transcripts. PolV-dependent transcripts originate from the same regions as P4 RNAs, measure around 200bp long and have a cap structure at their 5′ end but lack the 3′ poly(A) tail (Wierzbicki *et al*., 2008). PolV transcription involves the DDR complex (Law *et al*., 2010), which contains DEFECTIVE IN RNA DIRECTED DNA METHYLATION 1 (DRD1) (Kanno *et al*., 2004), a SNF2 chromatin remodelling factor, which acts in cooperation with RDM1, DEFECTIVE IN MERISTEM SILENCING 3 (DMS3) and the DMS11/GHKL ATPase (Lorkovic *et al*., 2012). This PolV transcript serves as a scaffold molecule to recruit the *de novo* DNA methyltransferase DRM2 by interacting with the AGO-siRNA complex factor (Wierzbicki *et al*., 2009) through a link made by RDM3/KTF1, a conserved PolII transcription elongation factor similar to SUPPRESSOR OF TY INSERTION 5 (SPT5) (He *et al*., 2009b). RDM12/IDN2 contains an XS domain known to bind dsRNA with 5′ overhangs and is therefore hypothesized to stabilize interactions between AGO bound 24-nt siRNAs and PolV-derived scaffold transcripts (Ausin *et al*., 2009). Very recently, IDN2 was also shown to mediate the interaction between PolV transcripts and SWI3B, a newly identified subunit of the SWI/SNF DNA remodelling complex, which regulates nucleosomes positioning (Zhu *et al*., 2013). IDN2 forms a complex with IDN2 PARALOG 1 (IDP1) and IDP2 (IDN2-IDP1/IDP2 complex) (Zhang *et al*., 2012) also identified as FACTOR OF DNA METHYLATION 1 (FDM1) and FDM2 in another study (Xie *et al*., 2012) (Fig. 1.3).

Besides the core components of the RdDM pathway described above, there are many factors that contribute to regulating RdDM activity or RdDM components. For example, many components of the splicing machinery were also shown to modulate RdDM. It is unclear whether mutations in the SR 45 splicing factor directly affect RdDM or the splicing of members of the RdDM pathway (Ausin *et al*., 2012). However, ZINC FINGER (ZnF) AND OCRE DOMAIN-CONTAINING PROTEIN 1 (ZOP1), MOS4 ASSOCIATED COMPLEX 3 (MAC3), MOS4, MOS12, MOS14 and several splicing factors such as PRECURSOR RNA PROCESSING (PRP3), PRP6 and PRP31 directly affect RdDM (Huang *et al*., 2013; Zhang *et al*., 2013; Du *et al*., 2015), indicating that the splicing machinery can participate to a certain extent in non-coding RNA processing at the RdDM target loci.

The activity of PolIV and PolV polymerases is regulated by INTERACT WITH RNA POLII 1 (IWR1)/RDM4/DMS4, a transcription factor conserved in yeast (He *et al*., 2009a; Kanno *et al*., 2010; Law *et al*., 2011). Consequently, *iwr1/rdm4/dms4* mutants show a reduced amount of PolIV-dependent primary 24-nt siRNAs, as well as a reduced amount of PolV-dependent intergenic transcripts, resulting in a loss of DNA methylation at the target loci. MORPHEUS' MOLECULE 1 (MOM1), which shares homology with the ATPase domain of the SWI2/SNF2 chromatin remodelling proteins, also regulates TGS via a complex interplay with polymerases PolIV or PolV (Yokthongwattana *et al*., 2010). PolV activity also requires the action of TOPOISOMERASE ALPHA (TOP1α), via the release of DNA topological tension generated by transcription (Dinh *et al*., 2014). Lastly, PolIV and PolV recruitment depends on factors that modify histones. Indeed, PolIV recruitment requires SHH1, which preferentially binds methylated H3K9 but not methylated H3K4 (Law *et al*., 2011; Law *et al*., 2013), whereas PolV requires SUVH2 and SUVH9 histone methyltransferases proteins for its recruitment to methylated DNA, through their SRA (SET and RING associated) domains. However, this recruitment does not depend on the histone modification activities of SUVH2 and SUVH9 because these two enzymes lack methyltransferase activity. Rather, immunoprecipitation experiments showed that SUVH2 could interact with the DDR complex (Johnson *et al*., 2014) (Fig. 1.3).

DNA methylation is also influenced by chromatin factors. Histone modifications actually play a role in the DNA methylation maintenance through self-reinforcing loops between DNA methylation and histone methylation, mostly through the action of the histone methyltransferases SUVH2, SUVH4 (also called KYP), SUVH5 and SUVH6 (Jackson *et al*., 2002; Ebbs *et al*., 2005; Ebbs and Bender, 2006). For example, the histone methyltransferase SUVH4 is recruited to CHG methylation through its SRA domain and, in turn, CMT3 binds SUVH4-derived methylated H3K9 through its chromodomain, thus reinforcing CHG methylation (Johnson *et al*., 2007). On the other hand, other histone-modifying enzymes remove active marks, thus promoting H3K9 methylation. Such histone-modifying enzymes include the histone deacetylase HDA6 (Aufsatz *et al*., 2002), the histone demethylase JMJ14 (Deleris *et al*., 2010) and the LYSINE-SPECIFIC HISTONE DEMETHYLASE 1 (LDL1) and LDL2 (Greenberg *et al*., 2013). Finally, deubiquitylation of Histone 2B by UBP26 is required for heterochromatic histone H3 methylation and DNA methylation (Sridhar *et al*., 2007).

Chromatin organization also plays a role in RdDM, independently of DNA methylation and histone modifications. For example, TEs are upregulated in *morc1*

and *morc6* single or double mutants, but 24-nt siRNAs accumulation, DNA methylation and H3K9 methylation are unaffected. These mutants are characterized by a decondensation of chromocenters (Moissiard *et al*., 2012; Moissiard *et al*., 2014). Similarly, MOM1 affects RdDM independently on DNA methylation and histone modifications. However, it is likely to act differently from MORC1/6 because *mom1 morc6* double mutants show synergistic effects with *mom1* on common loci (Moissiard *et al*., 2014).

Finally, the maintenance of methylation and TGS at RdDM targets is counterbalanced by several demethylating DNA glycosylases, including DEMETER (DME), DME-LIKE 2 (DML2) and DML3 (Penterman *et al*., 2007; Ortega-Galisteo *et al*., 2008). Moreover, active demethylation occurs through ROS1, which is guided by RNAs bound to the RNA-binding protein ROS3 (Zheng *et al*., 2008). DNA demethylation can also be mediated through ROS4/IDM1, an acetyltransferase protein that binds to unmethylated histone H3K4, and generates acetylated marks on histone H3 (Qian *et al*., 2012).

The majority of endogenous loci naturally targeted by RdDM are transposons and repeats. Nevertheless, a small fraction of protein-coding genes (PCG) produce 24-nt siRNAs, and this raises the question whether they contribute to regulating gene expression. Interestingly, 13% of the loci producing P4R2 RNAs that are regulated by RTL2 are located on PCG. DNA methylation and 24-nt siRNAs inversely correlate with mRNA accumulation at such loci, indicating that RdDM participates in modulating gene expression, at least at RTL2-regulated loci (Elvira-Matelot *et al*., 2016a). DNA viruses also are targeted by RdDM. For example, like other plant viruses, geminiviruses are targeted by RNA silencing; but, unlike RNA viruses, the viral genome is targeted by small-RNA-directed methylation. This is probably because geminiviruses produce double-stranded DNA intermediates that associate with cellular histone proteins to form minichromosomes. Mutations in AGO4, CMT3, DRM2, SUVH4 or PolIV increase sensitivity to geminivirus infection, indicating that plants use chromatin methylation as a defence against DNA viruses. Nevertheless, geminiviruses counteract this defence by expressing proteins that interfere with the proper functioning of the plant DNA methylation cycle (Raja *et al*., 2008).

1.4.2 DDM1/CMT2 pathway

Extensive CHH methylation is still observed in mutants impaired in PolIV-RdDM, implying that other pathway(s) contribute to maintaining CHH methylation. Such a pathway has recently been identified. It involves the SWI2/SNF2-Like protein DDM1 and the previously uncharacterized chromomethyltransferase CMT2. DDM1 is a chromatin-remodeller that binds nucleosomes and promotes nucleosome repositioning *in vitro* (Brzeski and Jerzmanowski, 2003). DDM1 has been identified as a critical factor for maintenance of DNA methylation as its loss of function leads to a 70% loss of methylation in TEs (Jeddeloh *et al*., 1999). DDM1 and RdDM were long considered as independent pathways, as they synergistically silence rDNA loci (Blevins *et al*., 2009). This hypothesis was recently confirmed by showing that DDM1 and RdDM, through DRD1, also have synergistic effects in silencing almost

all TEs in *Arabidopsis* (Zemach *et al*., 2013). These authors showed that residual CHH methylation in *ddm1* correlates with residual methylation observed in *cmt2*, and anti-correlated with that in RdDM mutants, thus identifying CMT2 as part of the DDM1 pathway. DDM1/CMT2 and RdDM target different TEs and domains, and thus act synergistically to silence almost all TEs in *Arabidopsis*. The DDM1/CMT2 pathway preferentially targets long heterochromatic TEs, whereas the RdDM pathway is more efficient in targeting preferentially short TEs located at the vicinity of genes, in euchromatic regions. This is in agreement with the fact that RdDM requires transcription by PolIV and PolV. Therefore, DDM1/CMT2 can counteract the influence of linker histone H1, thus favouring methyltransferases access to heterochromatic regions (Zemach *et al*., 2013) (Fig. 1.3).

1.5 Conclusions

Regulatory mechanisms have been put in place to control invading nucleic acids from endogenous (mainly transposons) or exogenous (mainly viruses) origins. Silencing is mediated by siRNAs, and can occur at either transcriptional or post-transcriptional level. From these defence mechanisms, specialized pathways have emerged to control certain endogenous genes through the action of new classes of small RNAs: miRNA, ta-siRNA or nat-siRNA. Interestingly, the development of transformation methods has revealed that transgenes are more prone to silencing than are endogenous genes, suggesting that the position and arrangement of genes within the genome is not random, and that genes cannot be moved around without perturbing expression.

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