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## Functional analysis of plant RNaseIII enzymes

Nahid Shamandi

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**Par**

**Nahid SHAMANDI**

**Functional analysis of plant RNaseIII enzymes**

**Composition du jury :**

<b>Directeur de thèse :</b>	<b>Hervé VAUCHERET</b>	<b>Directeur de Recherche (IJPB)</b>
<b>Rapporteur :</b>	<b>Cécile BOUSQUET-ANTONELLI</b>	<b>Chargé de Recherche (LGDP)</b>
	<b>Olivier VOINNET</b>	<b>Directeur de Recherche (ETH)</b>
<b>Examineur :</b>	<b>Moussa BENHAMED</b>	<b>Maitre de Conférences (IBP)</b>
	<b>Martin CRESPI</b>	<b>Directeur de Recherche (ISV)</b>

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## Abstract

Small RNAs, including miRNA and siRNA, play essential regulatory roles in genome stability, development and stress responses in most eukaryotes. Plants encode four DICER-LIKE (DCL) RNaseIII enzymes. DCL1 produces miRNAs, while DCL2, DCL3 and DCL4 produce diverse size classes of siRNA. Plants also encode RNASE THREE-LIKE (RTL) enzymes that lack DCL-specific domains and whose function is largely unknown.

Arabidopsis plants over-expressing RTL1 exhibit morphological defects and lack all types of small RNAs produced by DCL2, DCL3 and DCL4, indicating that RTL1 is a general suppressor of plant siRNA pathways. RTL1 activity requires a functional RNaseIII domain. RTL1 is naturally expressed only weakly in roots, but virus infection strongly induces its expression in leaves, suggesting that RTL1 induction is a general strategy used by viruses to counteract the siRNA-based plant antiviral defense. Accordingly, transgenic plants over-expressing RTL1 are more sensitive to TYMV infection than wild-type plants, likely because RTL1 prevents the production of antiviral siRNAs. However, TCV, TVCV and CMV, which encode stronger suppressors of RNA silencing (VSR) than TYMV, are insensitive to RTL1 over-expression. Indeed, TCV VSR inhibits RTL1 activity, suggesting that inducing RTL1 expression and dampening RTL1 activity is a dual strategy used by viruses to establish a successful infection.

Plants over-expressing RTL2 and *rtl2* mutants do not exhibit morphological defects and do not show major changes in the endogenous small RNA repertoire. However, RTL2 over-expression enhances the accumulation of exogenous siRNAs in transient assays, and this activity requires a functional RNaseIII domain. Therefore, it is possible that plant RTL2 processes certain substrates to facilitate the action of DCL enzymes.

# 1 - Introduction

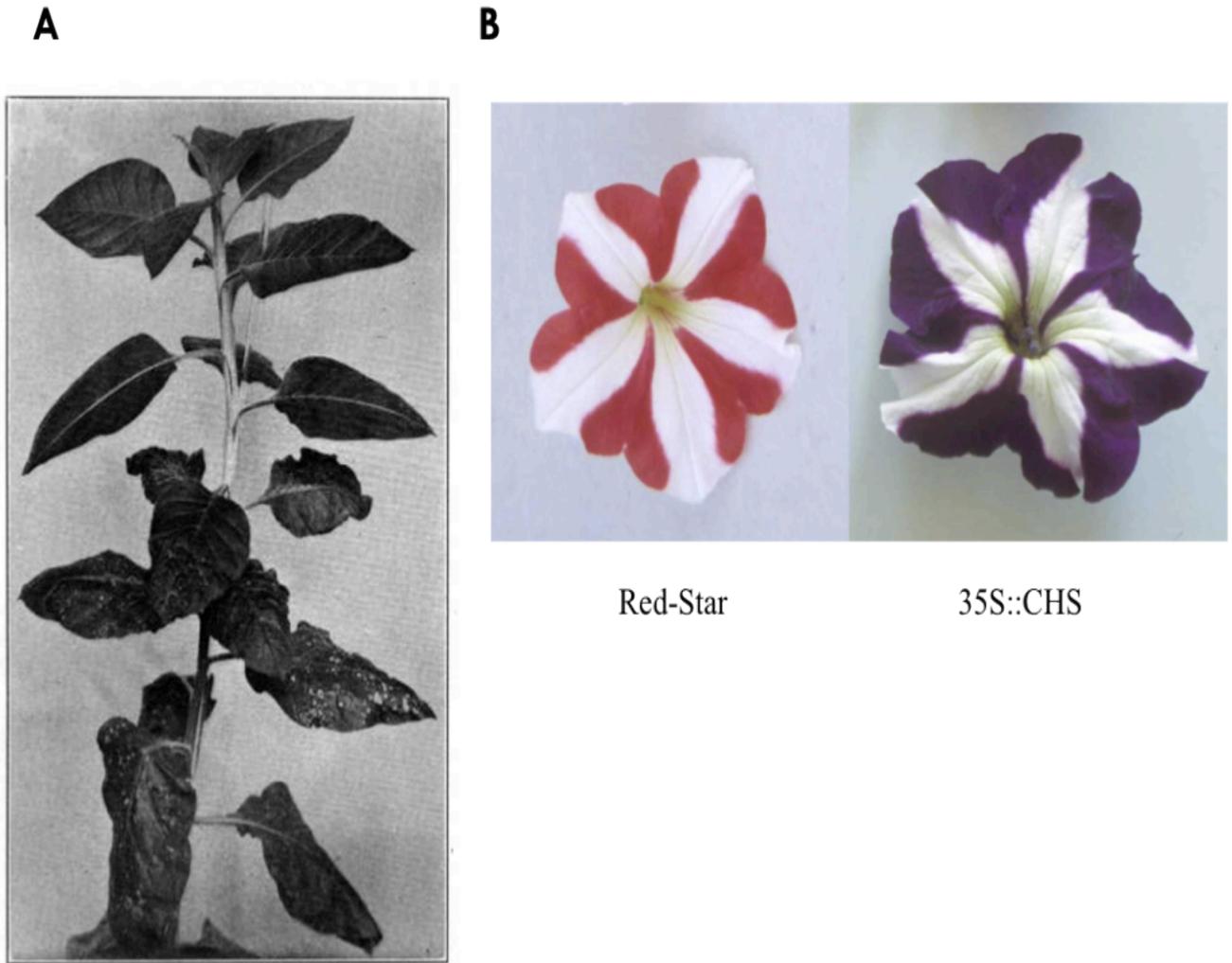
## 1.1 - RNA Silencing: Definition, History & Key factors

RNA silencing, also known as RNA interference (RNAi) is a conserved eukaryotic mechanism that regulates gene expression at transcriptional or post-transcriptional levels (Ghildiyal and Zamore, 2009; Lee et al., 2010).

The first RNAi phenomenon described in scientific literature dates back to 1928. In an article published in the *Journal of Agricultural Research*, S. A. Wingard reported that 23 days after inoculation of Turkish tobacco plants with ring-spot virus, only the leaves that had been specifically inoculated with the virus expressed symptoms of the disease while the others did not (Fig.1.1.A) (Wingard, 1928). In most leaves of the plants, a gradual decline and an eventual disappearance of the symptoms was observed. It was only in 1997 that Ratcliff et al. postulated that the recovery from viral infection involved specific targeting of viral RNAs by RNA silencing (Ratcliff et al., 1997).

In 1990 Napoli et al. observed another type of RNAi phenomenon, this time in petunia breeding (Napoli et al., 1990). Looking for new petunia varieties with deep purple petals, Napoli et al. cloned the petunia gene for chalcone synthase (CHS), which is a key factor in flavonoid biosynthesis during flower pigmentation pathways of petunia, under the cauliflower mosaic 35S promoter. Introduction of 35S::CHS in petunia resulted in an unexpected reduction in floral pigmentation - either a homogenous bleaching (up to full white) or variegated in sector/ring pigmentation patterns (Fig. 1.1.B) (Napoli et al., 1990). Molecular analyses showed that the level of endogenous and transgenic CHS in petunia transformants was 50-fold lower compared with control wild type plants. The same results were reported separately by Van der Krol et al. (van der Krol et al., 1990). Overall it was ascertained that RNA Silencing could not only suppress the expression of a transgene, but also can affect and reduce the expression of the homologous endogens of the host. This phenomenon was dubbed “co-suppression” (Napoli et al., 1990). Remarkably, the morphological and molecular phenotypes observed in 35S::CHS transgenic petunia plants resemble that of the petunia Red-Star variety (Fig 1.1.B) (Koseki et al., 2005). The Red-Star variety was obtained in 1830 through crosses between uniformly colored petunias and exhibits a duplication of the CHS genes, indicating that transgene-induced co-suppression mimics a natural manifestation of RNA silencing.

In animals the RNAi silencing mechanism was first reported by Guo and Kemphues (Guo and Kemphues, 1995). In an attempt to better understand the mechanisms of asymmetric cell division in *Caenorhabditis elegans*, Guo and Kemphues observed that the introduction of sense or antisense par-1mRNA, a protein kinase required for many aspects of early embryonic polarity, results in degradation of the endogenous par-1mRNA in *C. elegans*. In 1998, inspired by these findings Fire and Mello, suggested that it was double, not single, stranded RNA that was the trigger for gene silencing effect



**Fig. 1.1 Natural manifestations of RNA silencing**

**A)** Recovery from virus infection: Turkish tobacco plants inoculated with ring-spot virus exhibit symptoms in the bottom leaves and progressive recovery in the upper leaves. Top leaves are immune to re-inoculation (Wingard, 1928).

**B)** Petunia Red-Star : this variety was obtained in 1830 and results from crosses between uniformly colored petunia. It exhibits spontaneous CHS silencing due to the duplication of endogenous CHS genes (Koseki et al, 2005). The Red-Star silencing phenotype resembles that of transgenic 35S::CHS petunia plants (Napoli et al., 1990).

(Fire et al., 1998). They observed that the purified single stranded RNAs (ssRNAs) (sense or antisense) were significantly less effective than double stranded RNAs (dsRNA) in targeting the same mRNA (Fire et al., 1998). Indeed, ssRNA was effective only if the sense strand was injected into the animals, which will be hybridizing in to dsRNA *in vivo*. Although Fire and Mello provided the first explanations for previous paradoxical observations, yet the mechanisms by which dsRNA could cause the degradation of endogenous mRNA remained unclear.

In 1997, Palauqui et al showed that PTGS could be transmitted from a silenced root-stock to a non-silenced scions through a graft union (Palauqui et al., 1997). The same year, Voinnet and Baulcombe showed that the injection of dsRNA of a jellyfish green florescent protein (GFP) into one leaf of a plant carrying a GFP transgene led to the systemic silencing in the upper leaves of the infiltrated plants (Voinnet and Baulcombe, 1997). Still the nature of those signals was unclear as was the mechanism of their propagation and their specific targeting of mRNAs. Although it was generally thought that the antisense RNA molecules resulting from the unwinding of dsRNAs are main actors in detecting and binding to mRNA, the full-length antisense RNA was never detected in plants or animals exhibiting RNAi (Hamilton and Baulcombe, 1999). Hamilton and Baulcombe therefore speculated that although specific antisense RNAs are required for post-transcriptional gene silencing (PTGS), they still may be short and therefore hard to detect. They successfully detected the presence of 25-nt RNA in plants with PTGS activities and concluded that they were long enough to detect the specific sequences yet small enough to move through plasmodesmata and it was possible that they were components of the systemic signal and specificity of PTGS (Hamilton and Baulcombe, 1999). Shortly after, 21-23nt RNA was copurified with RNAi in *Drosophila* cells suggesting that dsRNA was converted to shorter intermediates - small interfering RNAs (siRNAs) - which can bind to their homologous mRNA targets and cause their cleavage (Hammond et al., 2000; Zamore et al., 2000).

Since then siRNAs have been discovered in every eukaryotic kingdom with the notable exception of *Sacharomyces cerevisiae* (Drinnenberg et al., 2009). Their crucial role in different pathways has become increasingly clear, nevertheless many more factors and enzymes associated with RNAi have been discovered and many more are still unknown.

## **1.2 - RISC: the main actor for RNA silencing**

The activity that targets and cleaves mRNAs is related to the action of RNA-induced silencing complex (RISC) (Song et al., 2004). Small interfering RNAs (siRNAs) and Argonaute proteins are the known signature components of RISC.

### **1.2.1 - Argonaute proteins**

To purify RISC, Tuschle et al. used a cell extract derived from human HeLA cells. They developed a technique for partially purifying RISC by conjugating the 3' termini of siRNAs to biotin, which

enabled coimmunoprecipitation of siRNAs with the associated protein complex (Martinez et al., 2002). Two proteins of ~ 100 kDa were identified that corresponded to Argonaute 1 and Argonaute 2 (Ago 1 and Ago 2) (Martinez et al., 2002).

Argonaute proteins are a highly conserved family that have been implicated in RNAi across several species (Carmell et al., 2002). Argonaute proteins are named after AGO1, the first discovered member, which was discovered in a forward genetic screen for genes involved in development (Bohmert et al., 1998). Argonaute proteins consist of four distinct domains: the N-terminal, PAZ, MID and PIWI domains (Hutvagner and Simard, 2008; Mallory and Vaucheret, 2010; Song et al., 2004).

The PAZ and Mid domains both help to anchor the small RNA guide: The PAZ domain binds to the 3' end using a series of conserved aromatic residues and the Mid domain provides a binding pocket for the 5' end (Hutvagner and Simard, 2008; Song et al., 2004). The Piwi domain contains an RNase-H-motif. Loading a highly complementary target into an Argonaute protein brings the scissile phosphate into the enzyme active site, which leads to the cleavage of the mRNA at the opposite nucleotides 10 and 11 of the small RNA guide. The PIWI domain cleavage products contain 3'-OH and 5'-phosphate, which is a characteristic feature of RNase-H-like processing (Hutvagner and Simard, 2008; Schwarz et al., 2004; Song et al., 2004).

AGO proteins are deeply conserved and are expressed in different tissues of almost all eukaryotes (Carmell et al., 2002). The exception being *Saccharomyces cerevisiae* which does not express Ago proteins and therefore does not appear to encode the small RNA pathway factors (Mallory and Vaucheret, 2010). In other species at least two Argonaute protein subfamilies exist: AGO and PIWI; and some references account a third subfamily: WAGO (Cheloufi et al., 2010; Faehnle and Joshua-Tor, 2007; Mallory and Vaucheret, 2010). The AGO subfamily is defined by similarity to *Arabidopsis thaliana* AGO1 (Bohmert et al., 1998; Cheloufi et al., 2010; Hutvagner and Simard, 2008). AGO proteins are found in both plants and animals and they have a common role in gene regulation. The PIWI subfamily is closely related to the *Drosophila melanogaster* PIWI protein (Hutvagner and Simard, 2008). PIWI proteins are animal-specific and forms part of an elegant innate immune system which can control the activity of mobile genetic elements (Malone and Hannon, 2009). The WAGO subfamily, the recently identified *Caenorhabditis elegans*-specific group, is specified to worms and acts in a variety of different biological process (Cheloufi et al., 2010; Faehnle and Joshua-Tor, 2007; Hutvagner and Simard, 2008; Yigit et al., 2006). Unlike the AGO and PIWI subfamilies the majority of WAGOs lack conservation of the DDH metal binding residues, suggesting a slicer-independent function (Faehnle and Joshua-Tor, 2007).

Although Argonaute proteins are present in bacteria, archaea and eukaryotes, the number of Argonaute genes that are present in different species varies. For instance, there are 8 proteins in humans (4 AGO and 4 PIWI), 27 in *C.elegans* (5 AGO, 3 PiWI and 19 WAGO), 10 AGO in *Arabidopsis thaliana*, 5 in

*D. melanogaster* (2 AGO and 3 PIWI) and only 1 Ago in *Schizosaccharomyces pombe* (Hutvagner and Simard, 2008; Mallory and Vaucheret, 2010).

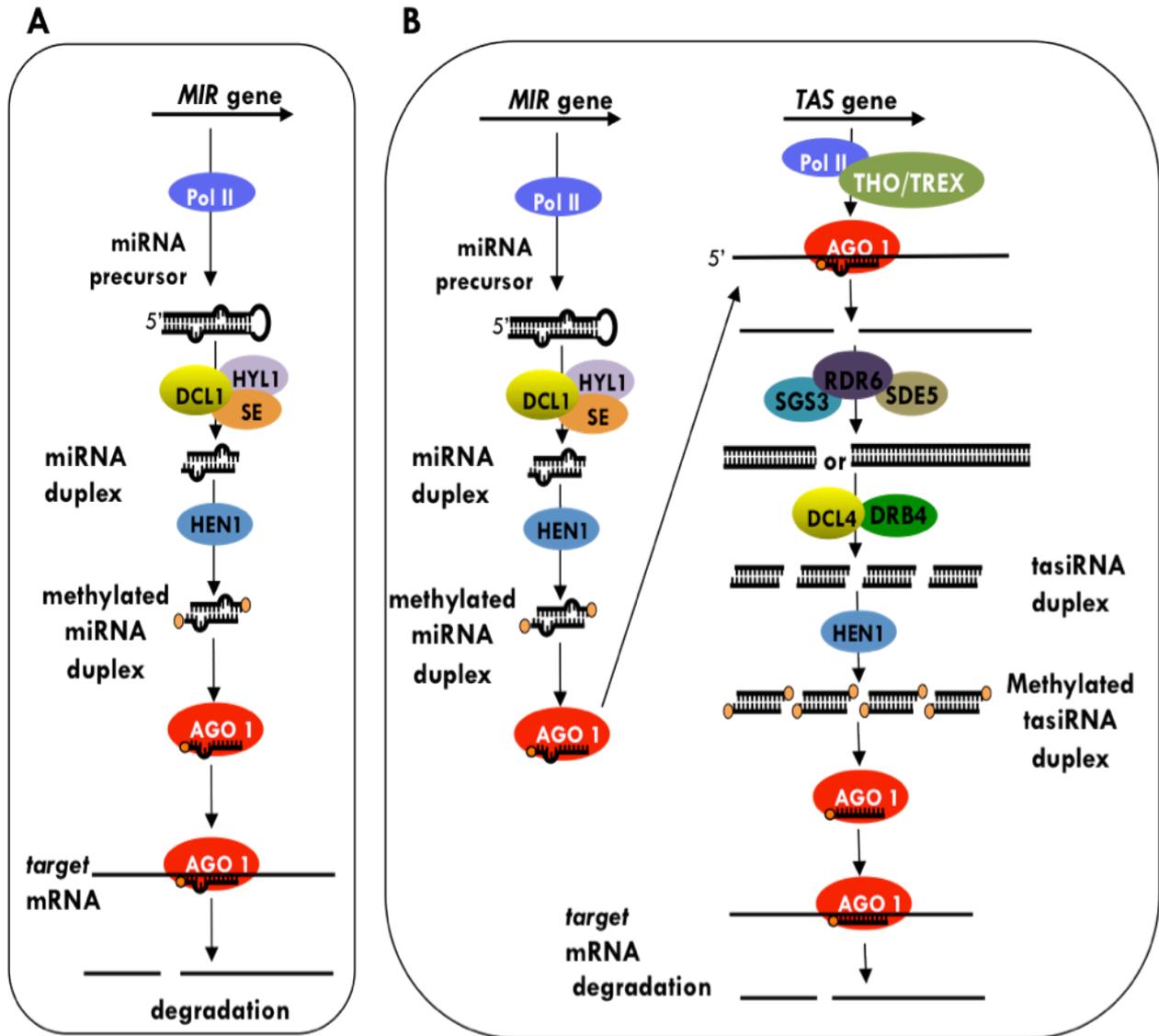
The association of small RNAs and Argonaute proteins is influenced by some specific factors. 5' nucleotide identity, small RNA length and mismatches in small RNA duplex all contribute to the sorting of small RNAs into specific AGO proteins (Forstemann et al., 2007; Kim et al., 2008). In *Arabidopsis*, AGO4, AGO6 and AGO9 mostly associate with 24-nt siRNAs whereas AGO1, AGO2, AGO5, AGO7 and AGO 10 are mostly associated with 21 and 22 nt molecules. AGO1 binds to sRNAs with 5'U while AGO2 prefers 5'A. AGO4, AGO6 and AGO 9 all bind to 24-nt siRNA, which mostly have 5'A, while AGO5 prefers 5'C (Aregger et al., 2012; Mallory and Vaucheret, 2010).

## 1.2.2 - Small RNAs

Small RNAs is the name using for small non-protein coding RNAs, 20 to 30 nucleotides (nt) in length, which play central roles in RNAi pathways (Baulcombe, 2004; Ghildiyal and Zamore, 2009; Mello and Conte, 2004). Different types of small RNAs exist which can recognise the mRNA target by base pairing interactions and cause either transcriptional inhibition or mRNA degradation in a post-transcriptional mode (Ghildiyal and Zamore, 2009). Small RNAs can also control histone modifications and chromatin formation (Buhler and Moazed, 2007). Different classes of small RNA have been found in eukaryotes which are different in their size, their origin and partially in their biogenesis (Castel and Martienssen, 2013). Most of small RNAs are produced by a specific cleavage of perfectly or imperfectly double-stranded (ds) RNA molecules by DICER or DICER-LIKE (DCL) proteins, which belong to the RNase III family of dsRNA specific endoribonucleases (Chapman and Carrington, 2007; Ghildiyal and Zamore, 2009). Following their production, small RNAs are loaded into specific AGO proteins (Hutvagner and Simard, 2008; Mallory and Vaucheret, 2010; Tolia and Joshua-Tor, 2007). Small RNAs guide AGO proteins to their target through complementary base pairing. Then, AGO (often associated with other proteins) silences these targets through RNA cleavage, translational interference, or chromatin modifications (Bartel, 2009; Brodersen and Voinnet, 2009). Plant small RNAs can be divided into two main classes: microRNAs (miRNA) and small interfering RNAs (siRNA), which comprise several subclasses (Castel and Martienssen, 2013; Vaucheret, 2006).

### 1.2.2.1 - miRNAs

miRNAs are the results of specific non-protein-coding *MIR* genes, which are transcribed by DNA-DEPENDENT RNA POLYMERASE II (Pol II). Pol II processes long single-stranded primary transcripts (pri-miRNA). Like regular protein-coding mRNAs, pri-miRNAs are capped at their 5' end, may contain introns, and are poly-adenylated at their 3' end (Jones-Rhoades et al., 2006). One of the specific and defining steps during miRNA biogenesis is the formation of a characteristic fold-back



**Fig. 1.2 miRNA and trans-acting siRNA pathways.**

**A)** miRNA pathway: Non-protein-coding *MIR* genes are transcribed by Pol II. Long single-stranded primary transcripts (pri-miRNA) fold-back and form a specific stem-loop structure. DCL1, HYL1 and SE protein are required for excision of miRNA/miRNA\* duplexes. Their 3' ends are methylated by HEN1 to protect them from degradation. Most miRNA are exported from the nucleus to the cytoplasm by HST. The two strands of the miRNA/miRNA\* duplexes separate, and the mature miRNA is loaded onto an AGO protein, mostly AGO1, to guide cleavage or translation repression of cognate target transcripts through sequence complementarity.

**B)** ta-siRNA pathway: Non-protein coding TAS genes are transcribed by Pol II, addressed by the THO/TREX complex to specific miRNA/AGO complexes. Clived TAS transcripts are protected from degradation by SGS3, while RDR6 and SDE5 generate dsRNA. These dsRNAs are diced by DCL4 and DRB4 into the 21-nt ta-siRNA duplexes, which are methylated by HEN1. One strand of the ta-siRNA duplex is loaded onto an AGO protein, mostly AGO1, to guide cleavage of cognate target transcripts through sequence complementarity.

stem-loop structure, which results from their transcript's intramolecular sequence complementarity (Brosnan and Voinnet, 2009; Vaucheret, 2006; Voinnet, 2009). In plants conversion of pri-to-pre-miRNA and then pre-to mature miRNA is catalyzed by the action of DCL1 (Kurihara et al., 2006). Besides DCL1, nuclear maturation and pri-to-pre-miRNA conversion also requires the presence of the double-stranded RNA-binding protein HYPOPLASTIC LEAVES1 (HYL1)(Han et al., 2004), the RNA binding protein DAWDLE (Yu et al., 2008), the CAP-BINDING PROTEINS CBP20 and CBP80/ABH1 (Gregory et al., 2008; Kim et al., 2008; Laubinger et al., 2008) and the C2H2-zinc finger protein SERRATE (SE)(Fig.1.2.A)(Lobbes et al., 2006; Yang et al., 2006).

In plants the dicing action of DCL1 and miRNA maturation occurs in the nucleus. The end result of the combined action of these proteins is a miRNA/miRNA\* duplex, which has typically two-nucleotide 3' overhangs (Jones-Rhoades et al., 2006). To protect the miRNA/miRNA\* duplex from degradation, its 3' ends are methylated by the methyltransferase HUA ENHANCER 1 (HEN1). Mutation in HEN1 results in 3'-end uridylation of miRNA and siRNAs, which leads to reduced accumulation (Li et al., 2005). After DCL1-mediated processing and HEN1-mediated methylation, most miRNA exit the nucleus and enter the cytoplasm. This exportation is facilitated by HASTY (HST), a member of the importin  $\beta$  family of nucleoplasmic transporters (Park et al., 2005). Upon export, the two strands of the miRNA/miRNA\* duplex are separated and the miRNA strand, also called guide strand, is bound by an AGO family protein. The miRNA governs the AGO protein for the target transcript recognition through sequence complementarity to the loaded miRNA. Thus, miRNAs act *in trans* upon the mRNAs of other genes to modulate their expression spatially and temporally. In the majority of studied cases, targeting leads to slicing of at least a fraction of total target RNAs (Fig. 1.2.A)(Mallory et al., 2008), although it is clear that suppression of targets involves both degradative and non-degradative mechanisms (Voinnet, 2009).

### **1.2.2.2 - siRNAs**

The majority of the small RNAs population in plants is represented short interfering RNAs, also known as siRNAs. There are a few key differences in the biogenesis of miRNA and siRNAs. siRNAs are derived from endogenous genes or inverted repeats, transgenes or viruses (Brodersen and Voinnet, 2006; Vaucheret, 2006). During their biogenesis they normally form a perfect double stranded RNA, which is then diced by the action of different types of DCLs. As a result of the different DCL enzymes, there are different sizes of 21, 22 or 24 nt for siRNAs. Mature siRNAs are loaded to different AGO proteins allowing them to have different functions. Based on their origin and their functions, the siRNA population in *Arabidopsis thaliana* can be divided to 5 subclasses: trans-acting siRNAs (ta-siRNAs), natural antisense transcript-derived siRNAs (nat-siRNA), endogenous IR-derived siRNAs (endo IR-siRNAs), PolIV/PolV siRNAs (p4/p5-siRNA) and NERD-siRNAs.

#### **1.2.2.2.a - ta-siRNAs**

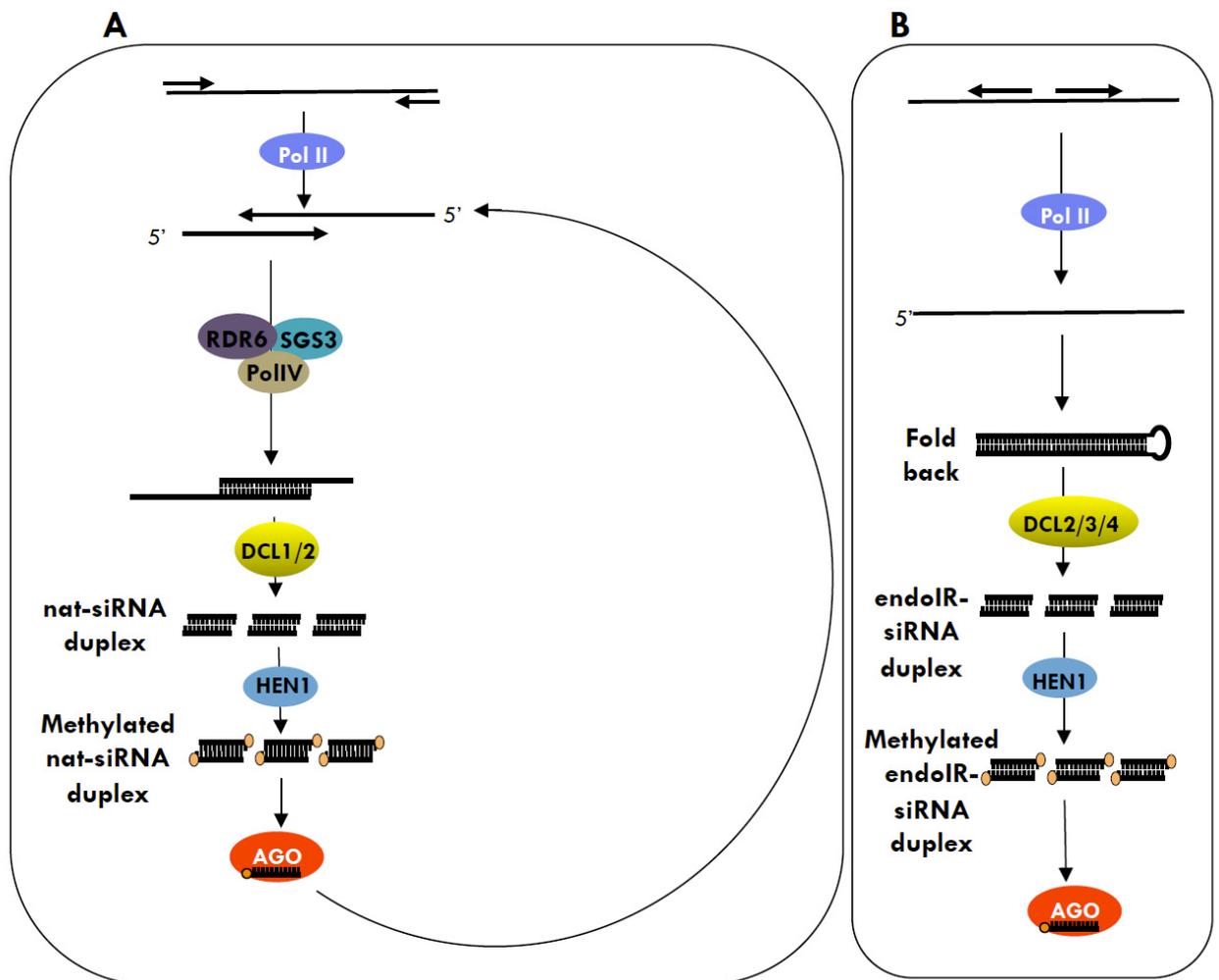
The ta-siRNA pathway is one of the most remarkable examples of overlapping between different small RNA pathways as its biogenesis requires components from both miRNA and siRNA biogenesis (Fig. 1.2.B). ta-siRNAs originate from non-protein coding TAS genes, which are transcribed by POL II (Allen et al., 2005; Peragine et al., 2004; Vazquez et al., 2004). The TAS transcripts contain a specific miRNA-binding site, they can therefore be recognized and sliced by a miRNA/AGO complex. The exportation of the primary transcripts to RISC complex depends on the THO/TREX complex (Jauvion et al., 2010; Yelina et al., 2010). Once the precursors are cleaved, they are protected from degradation by the action of SUPPRESSOR OF GENE SILENCING 3 (SGS3) (Elmayan et al., 2009). To generate a double-stranded RNA (dsRNA) from cleaved ssRNAs, both RNA DEPENDENT RNA POLYMERASE 6 (RDR6) and the putative RNA export factor SILENCING DEFECTIVE 5 (SDE5) are required (Hernandez-Pinzon et al., 2007; Yoshikawa et al., 2005). These dsRNAs are recognized by DCL4, which will then cut them into the 21-nt ta-siRNAs duplex (Fig. 1.2.B)(Gascioli et al., 2005; Xie et al., 2005).

This DCL4 dicing action requires the DOUBLE STRANDED RNA BINDING PROTEIN 4 (DRB4)(Nakazawa et al., 2007). Newly generated ta-siRNA also need to be methylated by HEN1(Li et al., 2005). One strand of the ta-siRNA duplex is loaded onto AGO1 to guide cleavage of target mRNA (Fig. 1.2.B)(Allen and Howell, 2010). ta-siRNAs seem to function mostly in developmental pathways like miRNAs (Vaucheret, 2005).

#### **1.2.2.2.b - nat-siRNAs**

Nat-siRNAs are derived from the convergent transcription of two overlapping sense and antisense transcripts (NAT), which can potentially form dsRNAs. These transcripts can originate from close genomic locus (cis-NAT genes) or from two distinct loci (trans-NAT genes) (Wang et al., 2005). This first overlapped double stranded RNA is processed into 24- nt nat-siRNAs due to the action of DCL2 (Borsani et al., 2005). Despite the fact that the dsRNA precursor of nat-siRNAs result from the overlapping between two transcripts, the presence of Pol IV, RDR6 and SGS3 seems to be essential for the accumulation of primary nat-siRNAs (Borsani et al., 2005). In reported examples of nat-siRNAs it has been shown that one of the overlapping genes is regularly expressed while the expression of the complementary strand is stress-induced (Borsani et al., 2005; Katiyar-Agarwal et al., 2006). Primary nat-siRNAs are loaded on to an unidentified AGO protein, which in follow directs the cleavage of the constitutively expressed transcript (Fig. 1.3.A).

At this stage, the products of this cleavage will be turned to dsRNAs via the actions of Pol IV, RDR6 and SGS3 (Borsani et al., 2005). DCL proteins produce the short 21-nt nat-siRNAs from dsRNAs, which then also needs to be methylated by HEN1 to be protected from degradation (Borsani et al., 2005). 21-nt nat-siRNAs are capable of down regulating their original constitutive expressed



**Fig. 1.3 Nat-siRNA and endoIR-siRNA pathways.**

**A) nat-siRNA pathway :** Overlapping sense and antisense transcripts (NAT) derived from convergent transcription can potentially form dsRNA. Pol IV, RDR6, SGS3 and DCL1 or DCL2 are required for the production of primary nat-siRNAs, which are methylated by HEN1. Primary nat-siRNAs are loaded on to an unidentified AGO protein, which cleaves one of the original transcript and initiate the formation of secondary nat-siRNAs.

**B) endoIR-siRNA pathway:** transcription of endogenous inverted repeat (IR) loci produce non protein-coding RNAs that fold back into perfect or near perfect dsRNA. DCL2, DCL3, or DCL4 process these dsRNAs into 21-, 22- or 24-nt siRNAs. 24-nt siRNAs target methylation of the locus of origin.

transcribed. This category of siRNAs seems to be important during the plant adaptation to either biotic (for ex. in response to *Pseudomonas syringae* infection) (Katiyar-Agarwal et al., 2006) or abiotic stresses (for ex. in response to salt stress) (Borsani et al., 2005).

#### **1.2.2.2.c – endoIR-siRNAs**

Genome-wide analyses of Arabidopsis revealed the existence of many discrete loci corresponding to inverted repeats (IR) of different sizes. The transcription of such IRs can fold back and produces perfect or near perfect complementary long dsRNA (Fig. 1.3.B).

DCL2, DCL3, DCL4 but not DCL1 can process these dsRNAs and produce siRNAs of all sizes referred to as endoIR-siRNAs (Fig.1.3.B). The evolution, genetics and biological implications of IR loci remain relatively unexplored. It has been proposed that their pathways might have important implications in adaptation to stress, epiallelism and epigenetic memory (Dunoyer et al., 2010).

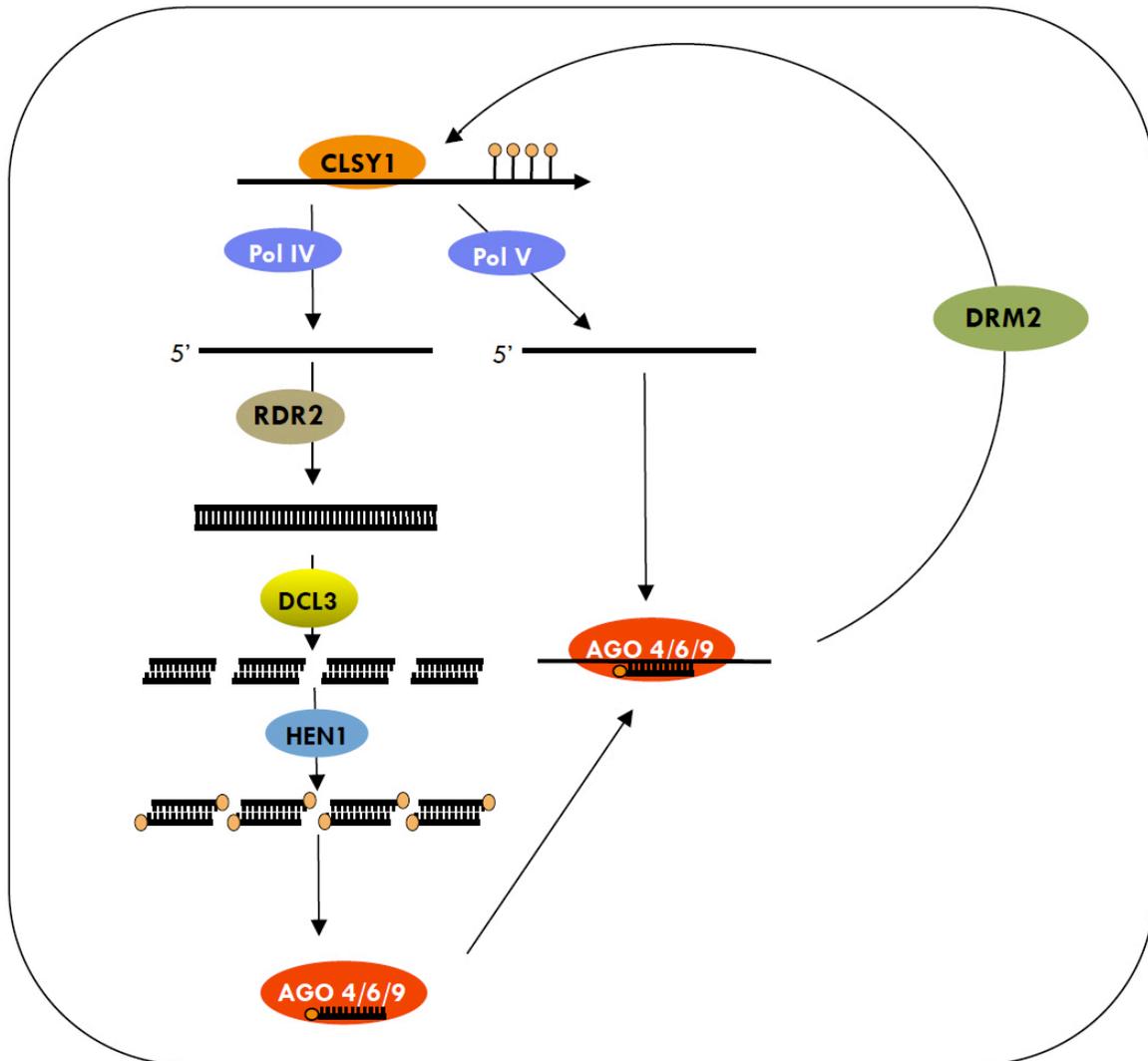
#### **1.2.2.2.d – p4/p5-siRNAs**

The majority of the small RNA population in plants are 24-nt which are mostly derived from transposons and DNA repeats (Kasschau et al., 2007). During their biogenesis, Pol IV is needed to transcribe specific genomic loci. The mechanism during which Pol IV is able to find its target genome remains largely unknown (Lahmy et al., 2010; Pikaard et al., 2008).

ssRNAs resulting from the action of Pol IV are transformed into dsRNA by RDR2 (Pikaard et al., 2008). It has been shown that the presence of a putative chromatin-remodeling factor, CLASSY1 (CLSY1) is essential during both the Pol IV and RDR2 processing steps (Dunoyer et al., 2010; Greenberg et al., 2011; Smith et al., 2007). Consequently, dsRNAs are sliced into 24-nt duplexes by DCL3. HEN 1 is also needed to methylate and protect these 24-nt siRNAs duplexes (Li et al., 2005). AGO4, AGO6 or AGO9 can interact with one of the strands of these duplexes to form RISC complexes that cleave transcripts produced by Pol V and address the *de novo* DNA methylase DRM2 to the corresponding loci (Fig. 1.4)(Haag and Pikaard, 2011).

#### **1.2.2.2.e - NERD siRNAs**

NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION (NERD)- siRNAs, are a newly discovered class of 21- and 22-nt siRNAs which appear to be unique to plants. The functional characterization of NERD siRNAs revealed the existence of an additional siRNA-mediated DNA methylation pathway, which silences a specific subset of genomic regions in Arabidopsis. Not only NERD siRNAs are shorter than classical 24-nt siRNAs, which mediate TGS, their processing requirements are also different from those of p4/p5-siRNAs. As it is obvious from their name, their biogenesis does not require RDR2 but RDR1 and/or RDR6 instead. Their processing into short siRNAs requires DCL4 and/or DCL2. Finally, they interact with AGO2 to bind to unmethylated



**Fig. 1.4 p4/p5-siRNAs pathway.**

24-nt p4/p5-siRNAs mostly derive from transposons and DNA repeats, which are transcribed by Pol IV at loci associated to CLSY1. ssRNAs resulting from the action of Pol IV are transformed into dsRNA by RDR2. Consequently, dsRNAs are diced into 24 nt duplexes by DCL3 and are methylated by HEN 1. 24-nt siRNAs associate with AGO4, AGO6 or AGO9 and PolV-derived non protein-coding transcripts, to guides the *de novo* DNA methyltransferase DRM2.

histone3 lysine4 (H3K4) as specific genomic target loci and direct DNA methylation (Pontier et al., 2012).

### **1.3 - Modes of action**

RNAi is based on the action of RISC complex. Once the small RNAs of different sizes are processed, one of the duplex strands is loaded onto one or more AGO proteins. The Arabidopsis genome encodes 10 AGO proteins (Mi et al., 2008; Montgomery et al., 2008; Takeda et al., 2008; Zhu et al., 2011). Base-pairing between the small RNA guide strand and the target RNA determines locus specificity, whereas the distinct activity of 24-nt vs. 21-22-nt small RNAs determines the type of silencing. Indeed, 24-nt siRNAs mediate transcriptional gene silencing (TGS) through DNA methylation and chromatin modifications while 21- and 22-nt siRNAs and miRNAs mediate post-transcriptional gene silencing (PTGS) through RNA cleavage and translational inhibition (Ding and Voinnet, 2007; Mallory and Vaucheret, 2009; Voinnet, 2009). A common set of enzymatic activities (RDRs, DCLs and AGOs) and mechanisms (synthesis of dsRNA, dicing into small RNA, and small RNA-directed RNA cleavage) are shared between TGS and PTGS pathways. However, the two processes have different outcomes as well as specific actors.

#### **1.3.1 - Transcriptional gene silencing**

TGS is a function of nuclear RNAi, which in plants and fungi can repress target genes at the transcriptional level by epigenetic modification of chromatin. Different studies have shown the transcriptional silencing in yeast, cytosine methylation in plants and genome rearrangement in ciliates. Plants use DNA methylation to modulate the expression of transposons and repeats. In plants, DNA methylation in the promoter region can lead to transcriptional inhibition while methylation in the coding parts do not generally affect the transcription (Mette et al., 2000; Sijen et al., 2001). The first example of the RNA-directed DNA methylation (RdDM) in plants was reported during viroid infection in tobacco. DNA methylation of a transgene sequence homologous to that of the viroid genome was observed (Stam et al., 1998; Wassenegger et al., 1994).

As mentioned before, p4/p5-siRNAs are the main actors in directing RdDM. The transcripts from Pol IV serve as substrate for siRNA generation. RNA Pol IV interacts with RDR2 to produce dsRNA, which then are diced into 24 nt-siRNAs by the action of DCL3 (Kasschau et al., 2007; Law et al., 2011). These 24-nt siRNAs are exported into the cytoplasm where they can be loaded to one or more AGO proteins. AGO4 is the first AGO protein that has been shown to play a role during RdDM but there are at least 3 out of 10 Arabidopsis Argonautes, which are involved in this pathway (Zilberman et al., 2003). In fact, AGO6 was also found to act in certain cases, additionally to AGO4 to guide DNA methylation and TGS (Zheng et al., 2007). Also it has been shown that AGO9 can interact with

24 nt siRNAs and is required in silencing repetitive genomic regions involved in heterochromatin formation (Duran-Figueroa and Vielle-Calzada, 2010).

Once the siRNA/AGO complex is formed, it will be imported to the nucleus and be guided to complementary pol V intergenic non-coding transcripts. The final result of this co-transcriptional silencing is the deposition of repressive cytosine methylation at the loci, which are transcribed by Pol V. In plants, DNA methylation occurs at cytosine bases in all CG, CNG (where N is any nucleotide) and CHH (any asymmetric site, where H is A, C or T) sequences. The genetic requirements for each kind of methylation are different and specific (Chan et al., 2005; Law and Jacobsen, 2010).

In general, plant DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) catalyses the *de novo* methylation at asymmetric CHH sites, whereas the maintenance of CG methylation is carried the maintenance of CHG methylation by CHROMOMETHYLASE 3 (CMT3)(Cao and Jacobsen, 2002; Matzke et al., 2009). At some RdDM loci, siRNAs are not detected in the absence of DRM2 which would suggest that a positive feedback loop is required for efficient siRNA dependent *de novo* methylation and gene silencing (Cao et al., 2003).

### **1.3.2 - Post transcriptional gene silencing**

Post-transcriptional gene silencing was first observed as an unexplainable co-suppression of the transgene and the homologous endogenous gene(s) in petunia and tomato (Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990). Although both sense and antisense transgenes can lead to PTGS, inverted repeat transgenes more frequently induce PTGS (Beclin et al., 2002; Vaucheret, 2006). In fact, the inverted repeat transgenes produce RNA that are able to fold back directly and form a long dsRNA while sense transgenes need the action of RDR6 and SGS3 to be converted into a dsRNA (Brodersen and Voinnet, 2006; Vaucheret, 2006). Transgene-derived and endogenous dsRNA are diced into different size of small RNAs, but only 21- and 22-nt small RNAs have been identified as effectors of PTGS (Brodersen and Voinnet, 2006; Chapman and Carrington, 2007; Hamilton and Baulcombe, 1999; Vaucheret, 2006). 21- and 22-nt siRNAs can act either *in cis* or *in trans* if they exhibit complementarity with mRNA targets produced from loci that are distinct from the locus of origin. Post-transcriptional regulation in plants involves either RNA cleavage or translational repression (Brodersen et al., 2008).

## **1.4 - RNAi as an Antiviral Response**

Since the discovery of small RNAs, their vital functions became more and more clear. It may prove difficult to find a biological function or process that is not influenced at least to some degree or in some cell type, by small RNAs. Indeed, small RNAs participate to plant development, to adaptation to biotic and abiotic stress and to transposon control (Law and Jacobsen, 2010; Navarro et al., 2006;

Robert-Seilaniantz et al., 2007; Vaucheret, 2006; Zhang et al., 2011). Moreover, RNA silencing participates to antiviral defense in plants.

### **1.4-1 - Actors of the antiviral RNA silencing**

Most plant viruses have an RNA genome and replicate through dsRNA intermediates, which can trigger RNA silencing (Ding and Voinnet, 2007). Indeed, DCLs likely process these intermediate dsRNA into primary viral siRNAs (v-siRNAs), which loaded onto a RISC complex, can mediate the cleavage of viral transcripts (Ruiz-Ferrer and Voinnet, 2009; Shimura and Pantaleo, 2011). DNA viruses do not replicate through dsRNA intermediates, therefore the v-siRNAs precursors are likely produced from transcription of both strands of the viruses (Chellappan et al., 2004).

All four plant DCLs participate to the production of v-siRNAs (Blevins et al., 2006; Moissiard and Voinnet, 2006), but mostly DCL2 and DCL4 produce v-siRNAs that target viral RNA for destruction (Bouche et al., 2006; Deleris et al., 2006). DCL2 antiviral activity is only a replacement of DCL4 in case of genetically compromising or suppressing of DCL4 by dedicated proteins called viral suppressors of RNA-silencing (VSRs) (see below).

AGO1 and AGO2 are able to bind v-siRNAs and thus are key component of antiviral RISC (Takeda et al., 2008; Zhang et al., 2006). Indeed, *ago1* mutants are hyper-susceptible to *cucumber mosaic virus* (CMV) (Morel et al., 2002), and AGO1 is up-regulated upon virus infection (Zhang et al., 2006). In addition, AGO2 provides a secondary antiviral mechanism that is important when the primary AGO1-mediated layer is not active (Harvey et al., 2011; Wang et al., 2011).

RDR proteins also participate to the antiviral response. Indeed, RDR6 and RDR1 are required for efficient antiviral response by converting cleaved viral transcripts into dsRNA for the production of secondary v-siRNAs (Garcia-Ruiz et al., 2010; Wang et al., 2010).

### **1.4.2 - Viral suppressors of RNA silencing**

Plant viruses encode suppressor proteins of RNA silencing as counter-defense mechanisms that influence their accumulation and spread in their host plants (Bivalkar-Mehla et al., 2011; Burgyan and Havelda, 2011; Harvey et al., 2011; Zamore, 2004). The various VSRs are able to target many effectors of the silencing pathway involved in viral RNA recognition, dicing, RISC assembly, RNA targeting and amplification. Most of the identified VSRs are multifunctional. Indeed, they often perform essential roles for viruses such as coat proteins, replicases, movement proteins and transcriptional regulators (Gillet et al., 2013; Hao et al., 2011; Sansregret et al., 2013).

The V2 protein of *tomato yellow leaf curl geminivirus* (TYLCV) competes with SGS3 for RNA binding, thus preventing the production of secondary v-siRNAs (Glick et al., 2008).

The *pothos latent aureusvirus* P14 protein inhibits the processing of dsRNAs into siRNAs by binding to dsRNA in a size-independent manner (Merai et al., 2006; Merai et al., 2005).

The *cauliflower mosaic virus* (CaMV) P6 is a viral transcriptional trans-activator protein, which has been recently described as a VSR factor. In fact, P6 is able to suppress RNA silencing by interacting with dsRNA-binding protein 4 (DRB4), which is required for DCL4 functioning (Haas et al., 2008).

The *cucumber mosaic virus* (CMV) 2b protein is able to physically interact with the PAZ domain and part of PIWI domain of AGO1, which inhibits AGO1 activity (Zhang et al., 2006).

The *turnip crinckle virus* (TCV) P38 protein is also able to directly interfere with AGO1 function by binding to the PIWI domain through its GW motif (Azevedo et al., 2010).

The P0 protein of the phloem-limited *poleroviruses* can perturb AGO1 activity in an indirect manner. This protein contains an F-box motif, which hijacks the host SCF ubiquitin-protein ligase (E3) to promote AGO1 degradation (Baumberger et al., 2007; Pazhouhandeh et al., 2006).

The *tombuvirus* P19, which is one of the most studied VSRS, prevents RNA silencing through binding double-stranded siRNA (Silhavy et al., 2002).

The *Beet yellows virus* p21 protein can suppress silencing by binding directly to v-siRNAs (Ye and Patel, 2005).

At last, *sweet potato chlorotic stunt virus* (SPCSV) encodes an RNaseIII that likely cleaves siRNA, including v-siRNAs, thus inhibiting antiviral silencing activity (Cuellar et al., 2009; Kreuze et al., 2005).

A summary of different types of VSRS and their effects on RNAi pathways can be found in Fig. 1.5.

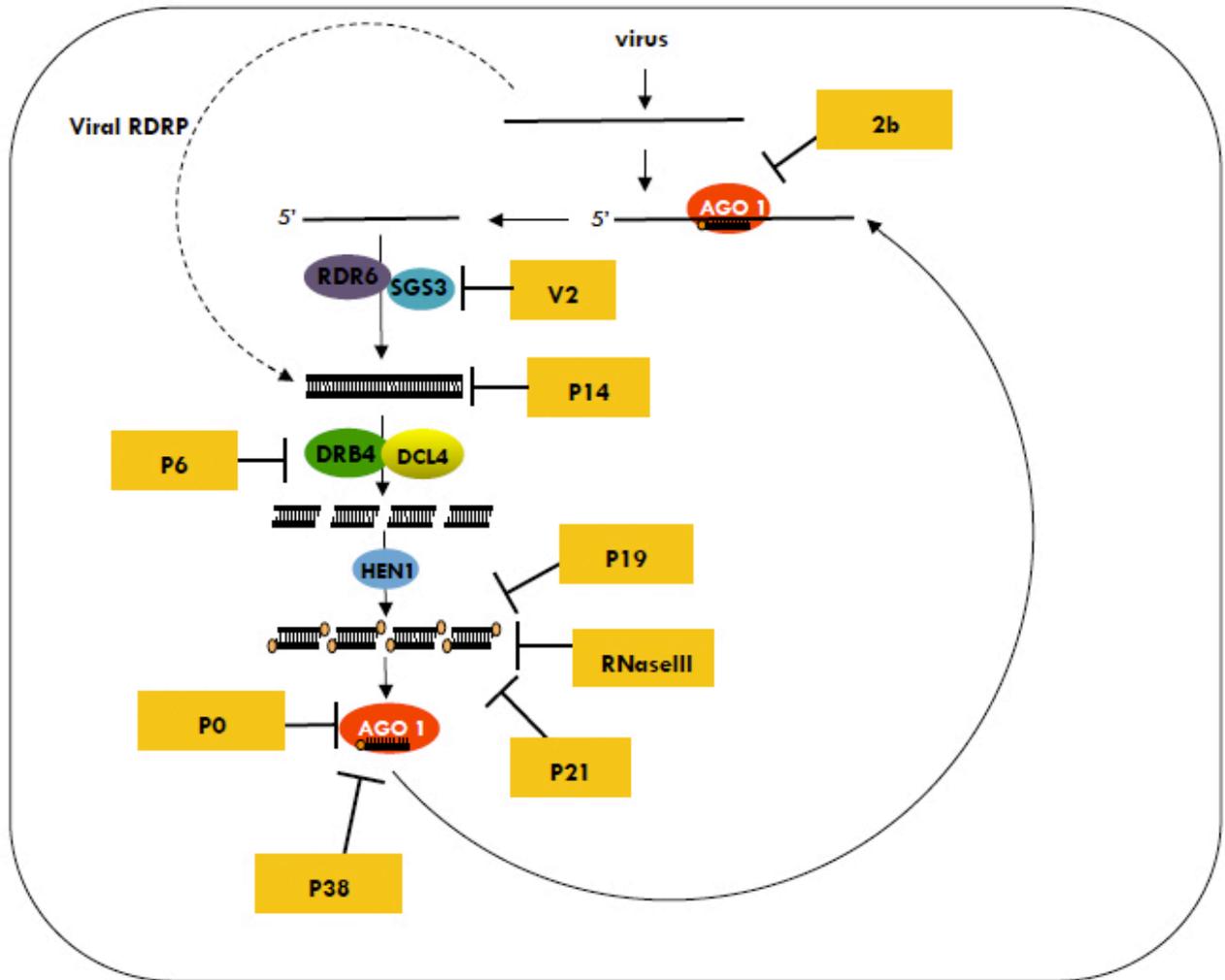
## **1.5 – Presentation of the objectives of the thesis**

### **1.5.1 - Dicer-independent small RNAs**

In eukaryotes, the biogenesis of small RNAs generally requires Dicer. However, a few Dicer-independent small RNAs have been reported in fungi, invertebrates and mammals (Cheloufi et al., 2010; Halic and Moazed, 2010; Lee et al., 2010).

In mammals, the conversion of pre-miR-451 to mature miR-451 requires the action of Ago 2 and not Dicer (Cheloufi et al., 2010). The mammalian Ago 2 protein is the only Ago that has endonuclease activity, and this activity is required for the production of miR-451 (Liu et al., 2004). Indeed, in vitro incubation of the wild type, but not catalytically inactivated, Ago2 with 5' end-labelled pre-miR451 produces mature miR-451 (Cheloufi et al., 2010).

In *Neurospora crassa*, a filamentous fungus, two dicer enzymes process dsRNAs into small RNAs, which are loaded onto the Argonaute protein QDE-2 (QUELLING DEFICIENT-2). QDE-2 interacts



**Fig. 1.5 Plant antiviral defense and viral suppressors of RNA silencing.**

A fraction of viral sense RNAs is translated into viral proteins. Another fraction is transformed into dsRNA by viral RNA-dependent RNA polymerases during viral replication. Viral dsRNA are diced by DCLs, mostly DCL2 and DCL4. Viral siRNAs associate with AGOs, mostly AGO1 and AGO2, to guide the cleavage of viral sense RNA. Cleaved viral products are protected from degradation by SGS3 and copied into dsRNA by cellular RNA-dependent RNA polymerases, mostly RDR6 and RDR1. dsRNA are diced by DCL2 and DCL4 to form secondary viral siRNAs, which reinforce the cleavage of viral sense RNAs.

Viruses encode proteins that act as suppressors of RNA silencing (VSR). These VSRs are able to inhibit many steps of the plant antiviral silencing pathway, including the protection of cleavage products by SGS3 (V2), the dicing of dsRNA by DCL4 (P14) and DRB4 (P6), the loading of siRNA duplexes onto AGO1 (P19, P21 and RNaseIII), the stability of AGO1 (P0), and the cleavage of viral sense RNA by AGO1 (2b).

with QIP, an exonuclease that removes the siRNA\* strand, resulting in an active RISC complex (Maiti et al., 2007). Sequencing results of QDE-2 associated small RNAs allowed identifying a distinct class of miRNA-like small RNAs (miRNAs). Both pre-miR and mature miR remain constant in the *dcl-1/dcl-2* double mutant, indicating that the biogenesis of miR is Dicer independent but requires QDE-2 (Lee et al., 2010).

In *Caenorhabditis elegans*, a substantial fraction of siRNA does not derive directly from input dsRNA. Instead, a population of antisense siRNAs called secondary siRNAs derives from the direct action of a cellular RNA-directed RNA polymerase (RdRP) on mRNAs that are targeted by primary siRNA (Sijen et al., 2007).

In the fission yeast *S. pombe*, a distinct class of small RNAs, called primal small RNAs (priRNAs), exists beside Dicer-dependent siRNAs. priRNAs are degradation products of abundant transcripts that result from bidirectional transcription of DNA repeats (Halic and Moazed, 2010)

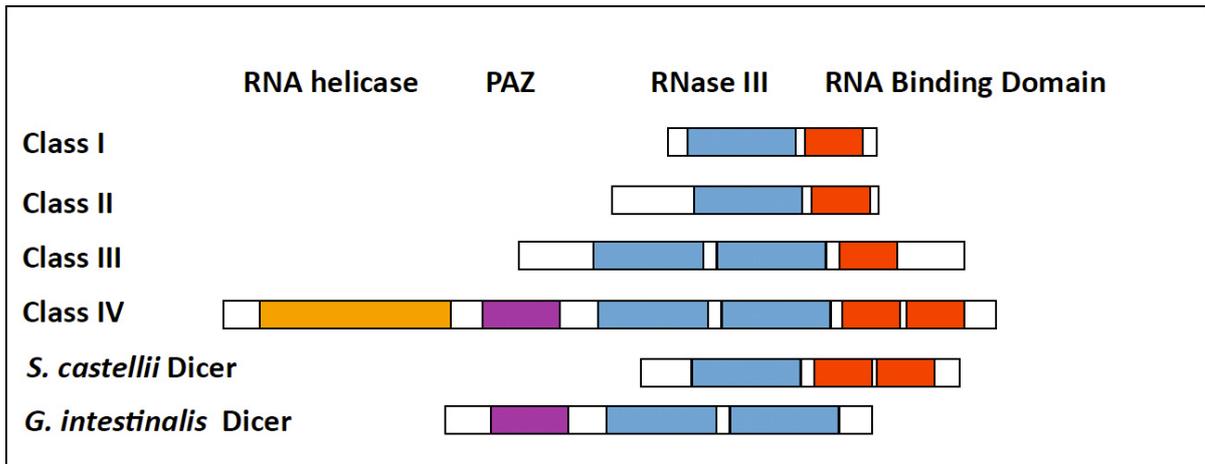
Up to now, Dicer-independent small RNAs have been only found in fungi, invertebrates and mammals, but not in plants. However, beside its four Dicer proteins, the Arabidopsis genome potentially encodes five non-Dicer proteins that are referred to as RNASE THREE-LIKE (RTL) proteins because they carry at least one RNase III domain (Fig. 1.6.B). So far, it has not been determined if these proteins participate to the small RNA repertoire, either by playing a direct role in small RNA production or an indirect role in regulating small RNA biogenesis or degradation.

### 1.5.2 - The RNaseIII family

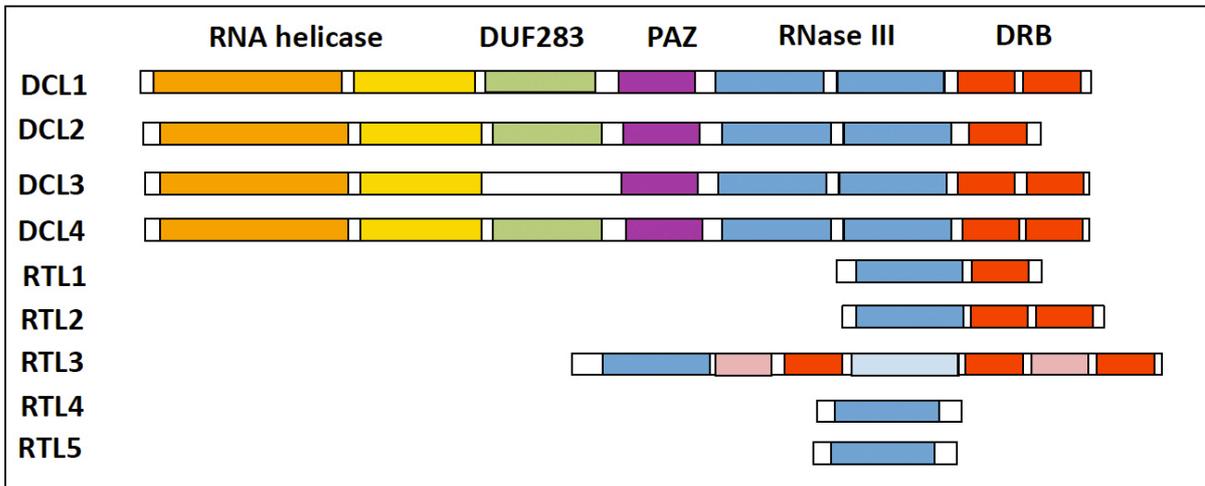
Double-stranded (ds)RNA-specific endoribonucleases referred to as RNaseIII enzymes, are found in all eukaryotes and bacteria (Comella et al., 2008). All members of the RNase III family are characterized by a highly conserved stretch of nine amino acid residues referred to as the RNaseIII signature motif (Comella et al., 2008; Du et al., 2008). RNaseIII enzymes range in length from approximately 200 to 2000 amino acid residues depending on the number of additional domains that they carry. Accordingly, they are divided in four classes based on their molecular weight and structural complexity (Fig. 1.6.A)(Filippov et al., 2000).

Class I has single RNase III and dsRNA-binding (DRB) domains (Kharrat et al., 1995). Enzymes of this class are found in bacteria and viruses. *Escherichia coli* and *Aquifex aeolicus* RNase III are representative enzymes of this class. Class II differs from Class I by the presence of a highly variable N-terminal domain extension of approx. 200 amino acid residues. *Saccharomyces cerevisiae* Rnt1 and *S.pombe* Pac1 enzymes are both members of class II (Lamontagne et al., 2004; Rotondo et al., 1997; Wu et al., 2004). Class III contains two RNase III domains, one DRB domain and a large N-terminal extension. Drosha, which processes pri-miRNAs to pre-miRNAs in animals, is a member of this class (Du et al., 2008; Filippov et al., 2000). Class IV is by far the largest and structurally most complicated

A



B



**Fig. 1.6 RNaseIII structures**

**A)** Canonical and atypical RNaseIII: *Escherichia coli* and *Aquifex aeolicus* RNaseIII are representatives of class I (one RNaseIII + one DRB). *Saccharomyces cerevisiae* Rnt1 and *S.pombe* Pac1 are representatives of class II (one RNaseIII + one DRB + N-terminal domain). Animal Drosha is representative of class III (two RNaseIII + one DRB). Animal and plant Dicer are representatives of class IV (one RNA helicase + one PAZ + two RNaseIII + one or two DRB). Atypical Dicer proteins lacking some of the Class IV domains are found in *S. castelli* and *G. intestinalis*.

**B)** Plant RNaseIII: Arabidopsis contains four DICER-LIKE (DCL) and five RNASE-THREE-LIKE (RTL) proteins. RTL1 resembles Class I proteins. RTL2 resembles atypical *S. castelli* Dicer. RTL3 exhibit an atypical organisation of multiple RNaseIII and DRB domains. RTL4 and RTL5 contain only one RNaseIII domain but no DRB domains.

RNaseIII family and corresponds to small RNA-producing Dicer enzymes, which generally contain two RNase III domains, one or two DRB domains, one PAZ domain, one RNA Helicase domain and one domain of unknown function (DUF283).

Class I and Class II enzymes, which carry a single RNase III domain, form a catalytic RNase III site by intermolecular homodimerization. The enzymatic studies on *Escherichia coli* RNase III have suggested a “single processing center model” for RNase III cleavage. In this model the action of RNaseIII proteins relies on the formation of a catalytic dimer of RNase III domains, which can be obtained by intermolecular homodimerization. Moreover, studies on the *Aquifex aeolicus* RNase III protein revealed that four patches of residues, also known as RNA-binding motifs1-4 (RBM1-4) are involved in protein-RNA interactions. RBM1 and RBM2 are located in the DRB domain whereas RBM3 and RBM4 are located in RNaseIII domain (Gan et al., 2006). The RNaseIII activity on dsRNA requires divalent cations such as  $Mg^{+2}$  or  $Mn^{+2}$  (Kreuze et al., 2005).

For the most part bacterial and eukaryotic RNase III cleavage of dsRNA precursors is performed in a non-sequence-specific manner. Nevertheless, the presence of AGNN or AAGU terminal tetraloops is a strong determinant for *S.cerevisiae* Rnt 1 binding and cleavage (Gaudin et al., 2006; Wu et al., 2004). RNaseIII products contain 5' phosphate and 3'hydroxyl termini and a 2-nt overhang at the 3' ends (Du et al., 2008). Generally, RNA cleavage mediated by Class I or Class II enzymes is performed in a non-sequence-specific manner. Nevertheless, the presence of AGNN or AAGU terminal tetraloops is a strong determinant for *S.cerevisiae* Rnt 1 binding and cleavage (Gaudin et al., 2006; Wu et al., 2004).

Class III and Class IV, which carry two RNase III domains, form a catalytic RNase III site by intramolecular heterodimerization between their two RNaseIII domains (Zhang et al., 2004). Class III Drosha proteins, which are involved in the first cut of animal miRNA precursors, are incapable of producing small RNAs. In contrast, Class IV Dicer proteins produce small RNAs. Dicer proteins contain one or two DRB domains. They also contain one PAZ, one RNA Helicase and a DUF283 domain. The PAZ domain recognizes the 3' 2-nt overhang of dsRNAs, while the RNA helicase act as a molecular ruler to measure the distance from the dsRNA end (recognized by the PAZ domain) (Liu et al., 2009). The DUF283 domain has recently been suggested to play significant roles for partner protein selection in small RNA processing (Fig. 1.6.B) (Qin et al., 2010).

Remarkably, some Dicer proteins from lower eukaryotes have simpler domain structures. For example, the *Giardia intestinalis* Dicer protein only contains a PAZ and two RNase III domains (Macrae et al., 2006). The PAZ domain is directly connected to the RNase IIIa domain by a long  $\alpha$  helix that runs through the handle of the molecule. This “connector” helix is encircled by the N-terminal residues of the protein, which form a platform domain composed of an antiparallel  $\beta$  sheet and three  $\alpha$  helices. Measuring from the active site of the first RNase III domain of *Giardia* Dicer to the 3' overhang-binding pocket in the PAZ domain gives a distance of ~65 Angstrom, which matches

the length of RNA fragments produced by *Giardia* Dicer, which are 25 to 27 nucleotides long (Macrae et al., 2006).

Moreover, *S. castelli* Dicer has one RNaseIII domain and two DRB domains, and thus differs from Class II Rnt1 by an additional DRB domain (Drinnenberg et al., 2009; Weinberg et al., 2011). Despite significant differences in domain structure between the budding-yeast and canonical Dicers, the RNase III signature motif and active site residues important for dsRNA cleavage are conserved in Dcr1, suggesting that the RNase III cleavage activity is conserved in Dcr1. In fact, *S. castelli* extract contains an activity that produces 22- to 23-nt RNAs from dsRNA precursors. Moreover, in *Δdcr1* *S. castelli* mutants, siRNAs failed to accumulate, suggesting that the Dcr1 functions in Dicer-specific activities (Drinnenberg et al., 2009). However, budding-yeast Dicers produce siRNAs through a mechanism different from that of canonical Dicers. Instead of successively removing siRNA duplexes from the dsRNA termini, Dcr1 starts in the interior and works outward (Weinberg et al., 2011).

### 1.5.3 – Functions of plant RNaseIII

The Arabidopsis genome encodes four Dicer-Like (DCL) proteins with known functions. DCL1 produces miRNAs from imperfectly double-stranded stem-loop RNA transcripts which are generated from MIR genes (Park et al., 2005). DCL2, DCL3 and DCL4 process near-perfect long dsRNA precursors to generate either siRNAs or young miRNAs. 24-nt p4/p5-siRNAs are derived from the action of DCL3 (Daxinger et al., 2009). DCL4 produces 21-nt young miRNA, ta-siRNAs and v-siRNAs during viral infections (Bouche et al., 2006; Gascioli et al., 2005; Xie et al., 2005). DCL2 produces 22-endoIR-siRNAs and can replace DCL4 and DCL3 in their absence (Bozorov et al., 2012; Deleris et al., 2006; Dunoyer et al., 2010).

Beside the four DCL proteins, the Arabidopsis genome potentially encodes five RNASE THREE-LIKE (RTL) proteins, which have not been well studied yet. RTL1 contains only one RNase III and one DRB domain. RTL1 is only expressed in Arabidopsis roots at very low levels, and its function is unknown. RTL2 contains one RNaseIII domain and two DRB domains. RTL2 is ubiquitously expressed. RTL2 cleaves the 3'External Transcribed Spacer (EST) of ribosomal 45S-pre-rRNA *in vitro* and *in vivo* (Comella et al., 2008). An *in vitro* assay has revealed that RTL2 processes dsRNA into 21-25-nt small RNAs (Kiyota et al., 2011). *rtl2* mutants do not show any obvious developmental defects (Comella et al., 2008). RTL3 is the biggest member of the RTL family and has two RNase III domain and three DRB domains. RTL3 mRNA has not been detected in any tested tissue (Comella et al., 2008). RTL4 contains only one RNaseIII domain but no DRB domain, which makes it similar to the *Bacillus subtilis* Mini-III structure (Redko et al., 2008). RTL4 is expressed in almost every tissue. *rtl4* mutants are impaired in male and female gametophyte formation; however, RTL4 function remains unclear (Portereiko et al., 2006). RTL5 contains only one RNaseIII domain but no DRB

domains. RTL5 is expressed in almost every tissue. RTL5 is similar to maize RNC1, which splices several chloroplastic group II introns (Watkins et al., 2007).

The aim of this project is to expand our understanding of RTL1 and RTL2 functions and to shed light on their potential interaction with plant small RNA pathways.

## **2 – Materials and Methods**

### **2.1 – Plants and viruses**

#### **2.1.1 – Plant material**

All *Arabidopsis* plants are in the Col-0 ecotype. Transgenic lines *L1* and *L2* carry a *35S::GUS* transgene silenced by PTGS (Elmayan et al., 1998). The *rtl2* mutant corresponds to line GABI\_568D10 (Comella et al., 2008).

#### **2.1.2 – Plant transformation**

##### **2.1.2.1 – Arabidopsis transformation**

400 ml of overnight culture at 28°C of *Agrobacterium tumefaciens* C58C1 (pMP90) carrying plasmids of interest were centrifuged at 6000 rpm. The bacterial pellets were suspended in 300 ml of 5% sucrose, MgCl<sub>2</sub> 10 mM and Silwet L-77 0,015%. Plants at the early flowering stage were then dipped in to this solution for 30 seconds. This process was repeated on the same plants 6 days later. T1 plants, which carry the T-DNA, were identified on selective medium. Selected plants were kept in standard long-day conditions (16h of light and 8 h of dark at 20°C). 14-day old seedlings were used for majority of GUS enzymatic activity testes while 3-week old *Arabidopsis* leaves and/or flowers were used for different Northern and Western analyses.

##### **2.1.2.2 – *Nicotiana benthamiana* Agroinfiltration**

10 ml of overnight culture at 28°C of *A.tumefaciens* C58C1 (pMP90) carrying plasmids of interest were centrifuged at 6000 rpm. The bacterial pellets were suspended in a 10mM MgCl<sub>2</sub>, 10mM MES pH5.2, 150 mM acetosyringone solution to a final OD<sub>600</sub> of 1. The bacterial solution was then incubated at RT for 3 hours after which a final OD<sub>600</sub> of 0,1 was used to infiltrate the *N. benthamiana*'s leaves. These leaves were harvested 3 days after Agroinfiltration and used for the RNA extraction.

#### **2.1.3 – Virus and Bacterial inoculation**

##### **2.1.3.1- Virus inoculation**

10-day old in vitro plants were inoculated with *Turnip Crinkle Virus* (TCV), *Cucumber Mosaic Virus* (CMV), *Turnip Vein-Clearing Virus* (TVCV) or *Turnip Yellow Mosaic Virus* (TYMV) one day after transfert to soil. Virus inoculations were performed as previously described (Bouche et al., 2006; Mourrain et al., 2000). Plants were kept for 3 weeks in controlled-environment chambers (14h of

Table 2.1. Oligonucleotide sequences

Name	DNA Sequence
Oligonucleotides used for RT and PCR amplification	
attB1RTL1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGGATCGCAACTCTCAA
attB2RTL1R	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAGGTCTCCGAAAAATTTAGC
attB1RTL2F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGATCACTCTATCTCACC
attB2RTL2R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGATAATGAGATTTTCTCAAGG
attB2RTL1-R3-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTCTAAATGTATTATTGGCTCCAAC
attB1amiRF	GGGGACAAGTTTGTACAAAAAAGCAGGCTCTGCAGCCCCAACACACGC
attB2amiRR	GGGGACCACTTTGTACAAGAAAGCTGGGTATCCCCCATGGCGATGCTGCCTT
attB2RTL2-DRB1-R	GGGGACCACTTTGTACAAGAAAGCTGGGTGAATCTCTACACTATCTTCAT CAATAACC
attB1AT3G18145-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGCGGCTACGAAAGCTCTGG
attB2AT3G18145-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAAGTGCTTCGTTTAAATCTTTTCATAAGC
RTL1-6	GTTCTGTCATTGGATGGCTC
RTL1-7	CCAAAACCATCGGATCAGCGG
RTL1m2-F	GAATCGTACGCACTCCTAGAACTTTTAGGAGCTTCGATCC
RTL1m2-R	GGATCGAAGCTCCTAAAAGTTCTAGGAGTGCGTACGATTC
RTL2m-F	CCT TCT TAC GCG CGG CTA GAG TTC ATA GGC GCC AGT GCT ATT GG
RTL2m-R	CCA ATA GCA CTG GCG CCT ATG AAC TCT AGC CGC GCG TAA GAA GG
Oligonucleotides used as probes for small RNA detection	
miR159	TAGAGCTCCCTTCAATCCAAA
miR160	TGGCATAAGGGAGCCAGGCA
miR173	GTGATTTCTCTCTGCAAGCGAA
miR167	TAGATCATGCTGGCAGCTTCA
miR164	TGCACGTGCCCTGCTTCTCCA
miR156	GTGCTCACTCTCTTCTGTCA
miR839	GGGAACGATGAAAGGTTGGTA
amiR-RTL1A	AAGAGTGAATCGTACGAACTA
amiR-RTL1b	CGGGTCCCTGTAATCACCAA
TAS1	TACGCTATGTTGGACTTAGAA
TAS2	GTGCTTCACAATGCTCTTTC
siR1003	ATGCCAAGTTTGGCCTCACGGTCT
siR02	GTTGACCAGTCCGCCAGCCGAT
IR71	TCCTTTCCCTTCCCTTTCTAC
U6	TTGCGTGTATCCTTGCGCAGG
siRNA- At3g18145	AGTACGCTTCCCTTGACCAATCCA

light and 10 h of dark, at 20°C) after which the aerial parts of 4 to 12 plants were harvested, pooled separately for each treatment and used for RNA extraction.

### **2.1.3.2– Bacterial infection**

Arabidopsis plants were grown for 5 weeks in soil and were subjected to an 8 hour light and 16 hour dark cycle at 20°C, with 70% relative humidity. Leaves were infiltrated with bacterial suspensions of *Pseudomonas syringae* DC3000 AvrRpm1 or *Edwina amylovora* CFBP1430 at a concentration of  $10^7$  CFU per millilitre in sterile water or with sterile water (mock-inoculation), using a needleless syringe. At least 12 rosette leaves were used per condition for each biological replicate. These leaves were harvested 24 hours after infiltration with *E.amylovora* CFBP1430 and 3 days after infiltration with *P. syringae* DC3000 AvrRpm1. The infected leaves were grinded and then suspended in sterile water, which was used for serial dilutions and then plated on an LB medium. Bacterial colonies were counted 3 days later for each sample.

## **2.2 - Plasmid constructs**

### **2.2.1 – RTL1 and RTL2 tag fusion**

RTL1 and RTL2 genomic sequences (AT4G15417 and AT3G20420 respectively) were cloned into the pDONR207 (Gateway ® Technology- invitrogen) using attB1RTL1F, attB2RTL1R, attB1RTL2F and attB2RTL2R Gateway adapted primers (table 2.1). A series of LR reactions were performed to fuse RTL1 or RTL2 to Flag or Myc tags at their N- or C-terminus, under the control of the 35S promoter.

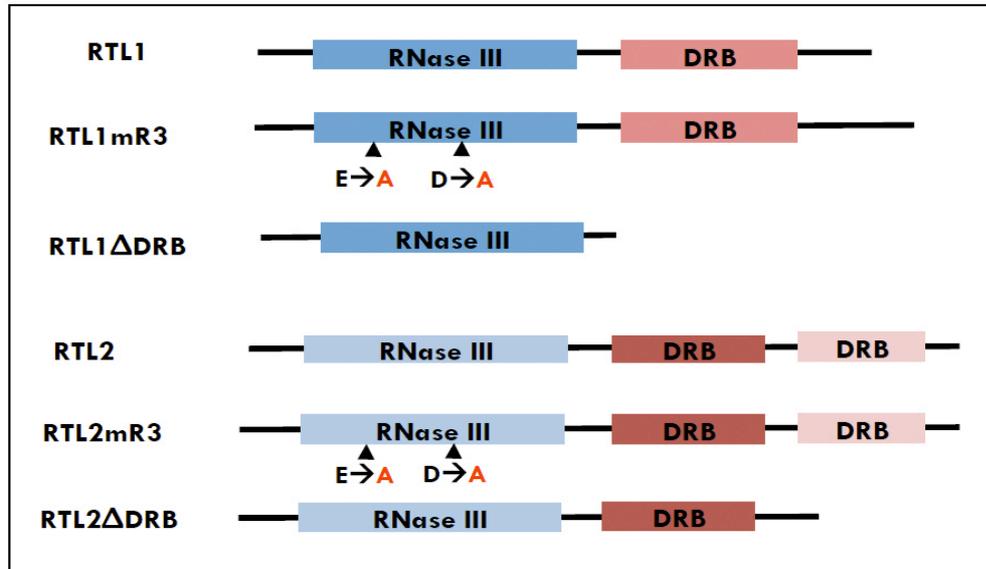
### **2.2.2 – RNaseIII mutation**

The point mutations in conserved amino acids of RNaseIII and/or DBR domains of RTL1 were done using a QuikChange ® Site-Directed Mutagenesis Kit. In the RNaseIII sequence, the amino acids 37 (Glutamin) and 44 (aspartic acid) were replaced by an Alanine using RTL6m2-F and RTL6m2-R primers for RTL1 and RTL2m-F and RTL2m-R primers for RTL2 (table 2.1).

### **2.2.3 – DRB deletion**

In order to generate the RTL1 $\Delta$ DRB, the RNase III sequence of RTL1 was amplified with attB1RTL1-F and attB2RTL1-R3-R primers (table 2.1). For RTL2  $\Delta$ DRB, the RTL2 RNaseIII and the first DRB domain sequences were amplified with attB1RTL2F and attB2RTL2-DRB-R primers. Each recombinant RTL was cloned in to the Gateway pDONR207 vector (Gateway ® Technology- invitrogen) and then transferred to the pGWB Gateway series under the control of the 35S promoter.

**A**



**B**

1	ATGGGATCGC	AACTCTCAAA	CGCAATCGAC	GGTAAAGATA	ACACCAAAC	50
51	CATCGGATCA	GCGGATCCGG	ACCAGCTTAT	GGAGATCGAA	TCCTTGGAGA	100
101	AGATCTTAAA	TTACAAGTTC	AAAGACAAGA	GTTTGTGCT	GAAAGCGTTC	150
151	ACAGACGCTA	GTTACGTTGA	TGACAAGAGT	GAATCGTACG	AACTCCTAGA	200
			TTCTCA	CTTAGCATGC	TTGAT	(amiR-RTL1a)
201	ACTTTTAGGA	GATTTCGATCC	TAAATATGGG	TATAATCTAT	GATTTTATTA	250
251	AGCTCTACCC	TAAAGAAGCA	CCTGGTCCGT	TGACCAAGCT	CCGAGCCGTC	300
301	AACGTAGATA	CCGAGAAATT	GGCACGGGTC	GCTGTAAATC	ACCAACTCTA	350
			GCCCAG	GGACATTTAG	TGGTT	(amiR-RTL1b)
351	TAGCTATCTC	CGTCATAAGA	AACCCTTGCT	TGAGGAGCAA	ATACTAGAAT	400
401	TTGTGGAAGC	CATGGAAAAA	TATCCATTAC	ATTCAAATGG	TCTTTTAAAA	450
451	GTTCCCTAAAG	TTCTAGCAGA	CATAGTAGAG	TCTACCATTG	GAGCTATCTT	500
501	CATGGACTGC	AACTCCACCG	AGACCGTCTG	GAAGGTGATA	AAACCATTGT	550
551	TGGAGCCAAT	AATACATTTA	GACAAAATGA	AGAGCCATCC	AATGACAGAA	600
601	CTCAATGAGA	TGTGTCAGAA	GAGGAACTTG	AAATTGACGT	CCAAAGACAC	650
651	TTGGGAAGAA	AATCAAACCT	ATTGTTTCCA	TATTGAGGAT	AAGTTTGTG	700
701	GATGTGGTCA	CCATCCTGTC	AAGAAAGAAA	CTGCTCGTAA	TTTCGCTGCA	750
751	AAAAATGCTA	TTGACAATTT	TGCTAAATTT	TTCCGGAGACC	TTTAG	795

**Fig. 2.1 Summary of RTL-derived constructs generated during this project.**

A) RTL1 and RTL2 genomic sequences from ATG to stop were cloned and then fused to a Flag or a Myc tag at their N- or C-terminus. For RTL1mR3 and RTL2mR3, the conserved E and D amino acids of the RNaseIII catalytic domain were replaced by A. For RTL1ΔDRB, the RNase III sequence of RTL1 was amplified. For RTL2 ΔDRB, the RTL2 RNaseIII and the first DRB domain were amplified.

B) Alignment of RTL1 coding sequence with amiR-RTL1a and amiR-RTL1b.

### **2.2.4 – RTL1 target**

The At3g18145 genomic sequence was cloned into the pDONR207 (Gateway ® Technology-invitrogen) using attB1At3g18145-F and attB2 At3g18145-R Gateway adapted primers (table 2.1) and then was transferred to pGWB2 Gateway vector.

### **2.2.5 – amiR-RTL1**

Two different amiRs, amiR-RTL1a and amiR-RTL1b were synthesized (Genscript) using the MIR319a backbone (Schwab et al, 2006). Sequence complementarity of amiR-RTL1a and amiR-RTL1b with RTL1 is shown in Fig. 2.1.B. Then by using Gateway adapted oligonucleotides attB1amiR-F and attB2-amiR-R we transferred these two constructs into the Gateway pDONR207 vector. This was followed by different LR reactions to construct vectors containing amiR-RTL1a and amiR-RTL1b under the control of the UBQ10 promoter.

A summary of all the generated constructs is available in Fig. 2.1.A.

## **2.3 – DNA, RNA and protein analysis**

### **2.3.1 – RNA extraction and Northern analyses**

For RNA gel blot analyses, frozen tissue was homogenized in a buffer containing; 0.1 M NaCl, 2% SDS, 50 mM Tris-Hcl (pH 9), 10 mM EDTA (pH 8) and 20 mM beta mercaptoethanol. RNAs were then obtained through a two-stage phenol extraction.

High molecular weight (HMW) and low molecular weight (LMW) RNA gel blot analyses were performed as previously described using 2 to 10 µg of the total RNA or HMW RNA extraction (Gy et al., 2007). A Fuji phosphor imager was used to quantify the hybridization signals where a U6 oligonucleotide probe was used for normalization of miRNA gel blot analyses. A list of all the probes, which were used in this study, is available in the table 2.1.

### **2.3.2 – RT reactions**

To synthesize cDNA, 2 µg of total RNA extractions were digested with DNaseI (Fermentas). 500 ng of final DNA-free RNA was used to do a reverse transcription with oligo-dT (Fermentas). Final PCR with Taq DNA polymerase (Thermo Scientific-fermantas) was then done on 5 µl of 1:5 dilutions of RT products using RTL1-6 and RTL1-7 primers (table 2.1). These results were standardized by comparing to the EF1α expression level.

### **2.3.3 – Protein extractions and immunoblotting**

Protein was extracted in a buffer containing 20 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM

MgCl<sub>2</sub>, 0.1% IGEPAL CA-630, 5 mM DTT, Sigma Protease Inhibitor. Protein concentrations were determined using a BioRad DC protein assay. 20 µg of protein was resuspended in a Laemmli buffer (20 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol, 40 mM DTT and 0.02% bromophenol blue), heated at 100 °C for 5 minutes, and separated on a 10% SDS-PAGE gel. Proteins were then transferred to PVDF membrane (BioRad). For detection, the membrane was blocked in 5% non-fat dry milk in 1× TBS, 0.1% Tween-20 (1×TBST) for 1 hour at room temperature, and incubated with a 1:1000 dilution of antiFlag or anti Myc primary polyclonal antibody (Agrisera) in 5% non-fat dry milk and 1× TBST for 1.5 hours at room temperature. The membrane was then rinsed in 1×TBST for 45 minutes before incubation with a secondary peroxidase-conjugated anti-rabbit antibody (Sigma) in 5% non-fat dry milk in 1×TBST at room temperature for one hour. After the membrane was rinsed in 1×TBST for 45 minutes, Flag and Myc signals were revealed using the Western Lightning kit (PerkinElmer Life Sciences) and the Immunstar WesternC kit (Biorad) as per the manufacturer's specifications.

### **2.3.4 – GUS enzymatic activity test**

GUS activity was quantified by measuring the quantity of 4-methylumbelliferone product generated from the substrate 4-methylumbelliferyl-b-D-glucuronide (Duchefa) on a fluorometer (Fluoroscan II; Thermo Scientific), and fluorescence values were normalized to total protein extracted, which was quantified using an absorbance microplate reader (Elisa Elx 808; Avantec) and the Bradford protein assay.

## 3 – Results

In eukaryotes, the biogenesis of most small RNAs depends on Dicer proteins that are the members of the Class IV of RNaseIII enzymes. The plant model *Arabidopsis* encodes four Dicer-like (DCL) proteins (Schauer et al., 2002). DCL1 produces ancient, conserved, miRNAs that average around 21-nt in length and derive from relatively short, imperfectly double-stranded stem-loop RNA precursors transcribed from non-protein coding MIR genes. By contrast, DCL2 and DCL3 produce siRNAs and DCL4 produces young miRNAs and siRNAs, all of which derive from long dsRNA precursors originating from either long inverted repeats (young miRNAs and some siRNAs) or from the action of RNA-dependent RNA polymerases (RDR) on single-stranded RNA precursor (some siRNAs). DCL2 produces 22-nt endoIR-siRNAs and viral/transgene siRNAs, DCL3 produces 24-nt PolIV/PolV-siRNAs and DCL4 produces 21-nt young miRNAs, ta-siRNAs and viral/transgene siRNAs (Bouche et al., 2006; Henderson et al., 2006). Most 21- and 22-nt small RNAs are loaded onto AGO1 to guide the cleavage of complementary mRNA (Voinnet, 2009), whereas 24-nt siRNAs associate with AGO4, which recruits PolV and DRD1, leading to transcriptional silencing through histone modification, DNA methylation and chromatin remodeling (Law and Jacobsen, 2010).

Aside from DCL1-4, the *Arabidopsis* genome has the capacity to encode five proteins referred to as RNASE THREE-LIKE (RTL), which mostly have unknown functions. RTL1, RTL2 and RTL3 harbor RNaseIII and DRB domains (Comella et al., 2008), whereas RTL4 and RTL5 contain only RNaseIII domains (Portereiko et al., 2006; Watkins et al., 2007).

### 3.1 – RTL1

RTL1 has one RNase III and one DRB domain. To gain insight into the biochemical function and biological roles of RTL1, the small RNA repertoire of *Arabidopsis* plants over-expressing RTL1 was compared to that of wild-type plants. We also investigated the importance of the RNaseIII and DRB domains in RTL1 function. Finally given the effect of RTL1 on siRNA accumulation we analyzed the biological role of RTL1 in plant-virus interactions.

#### 3.1.1 – Induction and suppression of RTL1 by viruses (article)

The results from this part of our study are collated in an article entitled “Plants encode a general siRNA suppressor that is induced and suppressed by viruses” which can be found below. As at the time of writing, this article has been submitted to the PLoS Biology journal.

## **Plants encode a general siRNA suppressor that is induced and suppressed by viruses**

Nahid Shamandi<sup>1\*</sup>, Matthias Zytnicki<sup>2\*</sup>, Cyril Charbonnel<sup>3,4</sup>, Gersende Lepère<sup>1</sup>, Allison C. Mallory<sup>1</sup>, Julio Sáez-Vásquez<sup>3,4</sup> and Hervé Vaucheret<sup>1</sup>

<sup>1</sup>Institut Jean-Pierre Bourgin, UMR 1318, INRA, Route de St-Cyr, 78000 Versailles, France.

<sup>2</sup>URGI, INRA, Route de St-Cyr, 78000 Versailles, France.

<sup>3</sup>CNRS, UMR 5096, LGDP, 66860, Perpignan, France.

<sup>4</sup>Univ. Perpignan Via Domitia, UMR 5096, LGDP, 66860, Perpignan, France.

\*These two authors contributed equally to this work

Author for correspondence: [herve.vaucheret@versailles.inra.fr](mailto:herve.vaucheret@versailles.inra.fr)

Short title: Induction and suppression of RTL1 by viruses

## **Abstract**

Small RNAs play essential regulatory roles in genome stability, development and stress responses in most eukaryotes. Plants encode DICER-LIKE (DCL) RNaseIII enzymes, including DCL1, which produces miRNAs, and DCL2, DCL3 and DCL4, which produce diverse size classes of siRNA. Plants also encode RNASE THREE-LIKE (RTL) enzymes that lack DCL-specific domains and whose function is largely unknown. Small RNA sequencing in plants over-expressing RTL1 or RTL2 or lacking RTL2 revealed that RTL1 over-expression inhibits the accumulation of all types of small RNAs produced by DCL2, DCL3 and DCL4, indicating that RTL1 is a general suppressor of plant siRNA pathways. By contrast, RTL2 plays minor, if any, role in the small RNA repertoire. *In vivo* and *in vitro* assays revealed that RTL1 prevents siRNA production by degrading dsRNA before they are processed by DCL2, DCL3 and DCL4. The substrate of RTL1 cleavage is likely long perfect (or near-perfect) dsRNA, consistent with the RTL1-insensitivity of miRNAs, which derive from short imperfect dsRNA. RTL1 is naturally expressed only weakly in roots, but virus infection strongly induces its expression in leaves, suggesting that RTL1 induction is a general strategy used by viruses to counteract the siRNA-based plant antiviral defense. Accordingly, transgenic plants over-expressing RTL1 are more sensitive to TYMV infection than wild-type plants, likely because RTL1 prevents the production of antiviral siRNAs. However, TCV, TVCV and CMV, which encode stronger suppressors of RNA silencing (VSR) than TYMV, are insensitive to RTL1 over-expression. Indeed, TCV VSR inhibits RTL1 activity, suggesting that inducing RTL1 expression and dampening RTL1 activity is a dual strategy used by viruses to establish a successful infection. These results reveal another level of complexity in the evolutionary battle between viruses and plant defenses.

**Author summary**

Most eukaryotes encode essential regulatory molecules called small RNAs. These molecules are mostly produced by a class of RNaseIII enzymes called DICER, which excise small RNA duplexes from long double-stranded (ds)RNA precursor molecules. Plants also encode several RNaseIII enzymes called RNASE THREE-LIKE (RTL) whose function is largely unknown. Here we show that RTL1 represses small RNA production by degrading dsRNA before they are processed by the DICER enzymes. RTL1 appears specific to a class of small RNAs called siRNAs but not to miRNAs, suggesting that it cleaves long perfect (or near-perfect) dsRNA but not short imperfect dsRNA. We found that RTL1 expression is induced after virus infection, which should favor infection by counteracting the plant anti-viral PTGS defense that is based on the production of siRNAs that target the virus for destruction. However, several viruses inhibit RTL1 activity, suggesting that a savant dosage between inducing and dampening RTL1 allows viruses to establish a successful infection.

**Blurb**

Plants encode an RNaseIII enzyme that is induced after virus infection and which represses siRNA production by degrading long dsRNA.

## Introduction

In eukaryotes, the biogenesis of small RNAs is either Dicer-dependent or Dicer-independent. Dicer-dependent small RNAs have been found in every eukaryotic kingdom (Bartel, 2009; Voinnet, 2009), with the notable exception of a few yeast species (Drinnenberg et al., 2011; Drinnenberg et al., 2009; Weinberg et al., 2011). In contrast, Dicer-independent small RNAs resulting from the action of RNA-dependent RNA polymerases, exoribonucleases, AGO proteins or a combination of these factors have been found in fungi, invertebrates and mammals but not in plants or protists (Brennecke et al., 2007; Cheloufi et al., 2010; Halic and Moazed, 2010; Sijen et al., 2007)

RNaseIII enzymes are double stranded (ds)RNA-specific endonucleases and are found in bacteria and eukaryotes. All members of the RNaseIII family contain a characteristic RNaseIII domain, which has a highly conserved stretch of nine amino acid residues known as the RNaseIII signature motif (Blaszczyk et al., 2001). RNaseIII proteins vary widely in length, from 200 to 2000 amino acids and have been subdivided into four classes based on their domain composition (Filippov et al., 2000). Class I is the simplest and the smallest, containing a single RNaseIII domain and a dsRNA binding domain (DRB); the bacterial and bacteriophage RNaseIII proteins belong to this class. Class II proteins, like class I, contain both an RNaseIII domain and a dsRNA binding domain but are distinguished from class I by the presence of a highly variable N-terminal domain extension and include the *S. cerevisiae* Rnt1 and *S. pombe* Pac1 proteins. Both of these yeast proteins are longer than bacterial RNaseIII proteins and contain an additional 100 amino acids at their N-terminus. Class III proteins have a DRB and two RNaseIII domains and include Drosha, which is involved in the first cut of miRNA precursors in animals but is incapable of producing small RNAs by itself. Class IV proteins correspond to animal and plant Dicer and contain an RNA helicase domain, a PAZ domain, either one or two RNaseIII domains and one or two DRB domains. Animal and plant Dicer proteins are the only RNaseIII proteins that have been shown to produce small RNAs in the size range of 18-24-nt, with the exception of class II RNaseIII from a few budding yeasts (Weinberg et al., 2011).

The plant model *Arabidopsis* encodes four Dicer-like (DCL) proteins (Schauer et al., 2002). DCL1 produces ancient, conserved, miRNAs that average around 21-nt in length and derive from relatively short, imperfectly double-stranded stem-loop RNA precursors transcribed from non-protein coding *MIR* genes. By contrast, DCL2 and DCL3 produce siRNAs and DCL4 produces young miRNAs and siRNAs, all of which derive from long dsRNA precursors originating from either long inverted repeats (young miRNAs and some siRNAs) or from the action of RNA-dependent RNA polymerases (RDR) on single-stranded RNA precursor (some siRNAs). DCL2 produces 22-nt endoIR-siRNAs and viral/transgene siRNAs, DCL3 produces 24-nt PolIV/PolV-siRNAs and DCL4 produces 21-nt young miRNAs, ta-siRNAs and viral/transgene siRNAs (Bouche et al., 2006; Henderson et al., 2006). Most

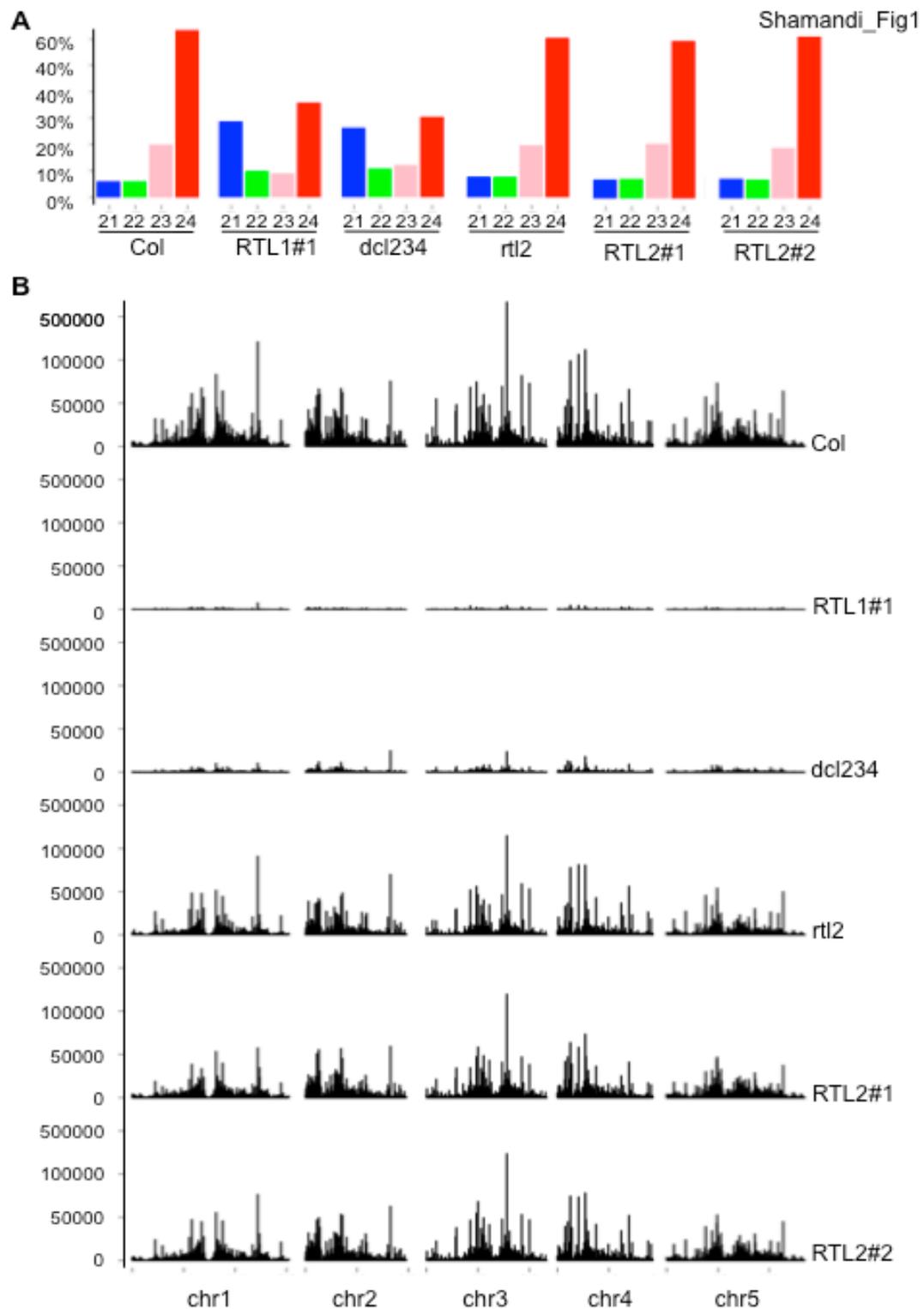
21- and 22-nt small RNAs are loaded onto AGO1 to guide the cleavage of complementary mRNA (Voinnet, 2009), whereas 24-nt siRNAs associate with AGO4, which recruits PolV and DRD1, leading to transcriptional silencing through histone modification, DNA methylation and chromatin remodeling (Law and Jacobsen, 2010).

Aside from DCL1-4, the Arabidopsis genome has the capacity to encode five proteins referred to as RNASE THREE-LIKE (RTL). RTL1, RTL2 and RTL3 harbor RNaseIII and DRB domains (Comella et al., 2008), whereas RTL4 and RTL5 contain only RNaseIII domains (Portereiko et al., 2006; Watkins et al., 2007). RTL1, which carries one RNaseIII domain and one DRB domain, is weakly expressed uniquely in roots (Comella et al., 2008). Mutants defective for RTL1 are not available in any of the public Arabidopsis stock centers, and RTL1 function is unknown. RTL2, which contains one RNaseIII domain and two DRB domains, is ubiquitously expressed. RTL2 processes, both *in vivo* as *in vitro*, the 3' External Transcribed Spacer (ETS) from ribosomal 45S pre-rRNA; however, mutants defective for RTL2 are viable and do not exhibit obvious developmental defects (Comella et al., 2008). RTL3 harbors two RNaseIII domains and three DRB domains, but its expression has not been detected in any tested tissue (Comella et al., 2008). RTL4 and RTL5 carry a single RNaseIII domain but lack DRB domains, and both genes are expressed in almost every tissue. Whereas the targets of RTL4 remain unknown, mutants defective in RTL4 are impaired in male and female gametophyte formation (Portereiko et al., 2006). RTL5 shares sequence similarity to maize RNC1, which is required for the splicing of several chloroplast group II introns (Watkins et al., 2007). Here, we show that RTL1 exhibits RNA cleavage activity, and that its over-expression prevents the accumulation of young miRNAs and all size classes of siRNAs, but does not affect ancient miRNA accumulation. Our results suggest that RTL1 processes long perfectly (or near-perfectly) paired dsRNA, thus preventing DCL2, DCL3 and DCL4 cleavage of these substrates. The possible roles of RTL1 in plant antiviral PTGS defenses also are discussed.

## Results

### *Plants over-expressing RTL1 lack ta-siRNAs, endoIR-siRNAs, p4/p5-siRNAs and young miRNAs*

To gain insight into the biochemical function and biological roles of RTL1 and RTL2, the small RNA repertoire of Arabidopsis plants over-expressing or lacking RTL1 or RTL2 was compared to that of wild-type plants. Plants over-expressing *RTL1* (*35S::RTL1* plants) exhibited consistent developmental defects; they remained small, had yellow and downward-curved rosette leaves, flowered late and were largely sterile. All progeny plants that carried the *35S::RTL1* transgene exhibited these developmental defects whereas siblings that had segregated the transgene away resembled wild-type plants, indicating that these developmental defects were directly caused by the presence of the *35S::RTL1* transgene. In



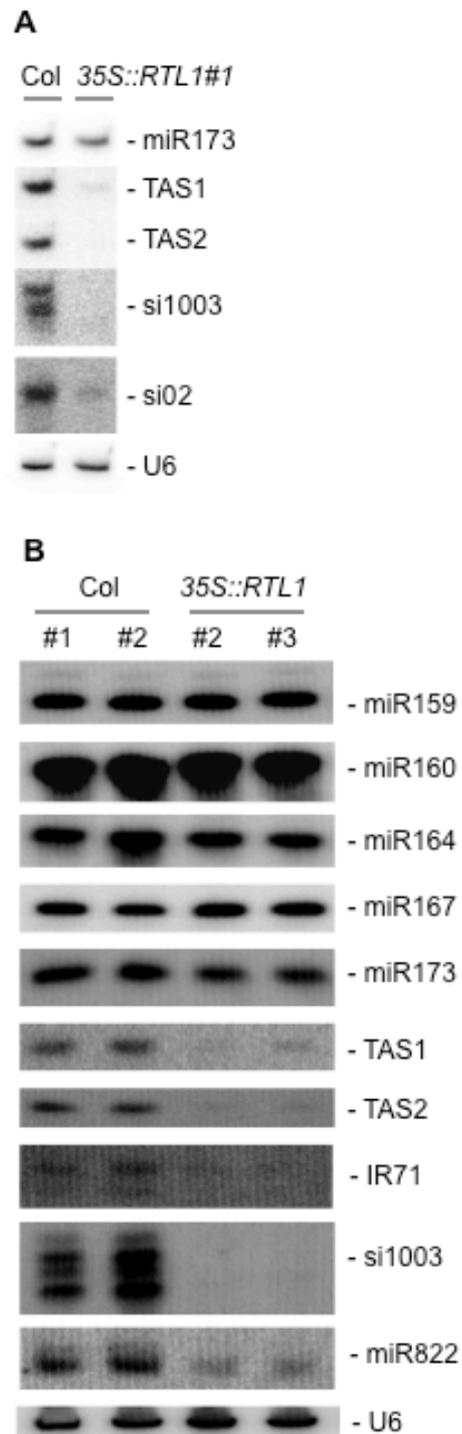
**Figure 1: Plants overexpressing RTL1 have reduced global levels of siRNAs.**

Small RNAs from wild-type (*Col*), *dcl2dcl3dcl4* and *rtl2* mutants and *35S::RTL1* and *35S::RTL2* transgenic plants were subjected to highthrough-put sequencing. Reads that match the Arabidopsis genome, excluding rRNA and tRNA matches, were retrieved. A) Size distribution of all reads, including unique and non-unique miRNAs and siRNAs. The proportion of each size of small RNA is indicated by a different color: 21-nt (blue), 22-nt (green), 23-nt (pink) and 24-nt (red). B) Normalized abundance of unique siRNAs spanning the nuclear genome. Normalization was made to the total of conserved miRNAs.

contrast, *35S::RTL2* plants did not exhibit consistent developmental defects under standard greenhouse growth conditions. One phenotypically representative *35S::RTL1* line and two independent *35S::RTL2* lines that accumulated high levels of *RTL2* mRNA were selected for genome-wide small RNA profiling. In addition, small RNAs were sequenced from a wild-type control, an *rtl2* loss-of-function mutant (Comella et al., 2008) and a *dcl2 dcl3 dcl4* triple mutant for comparison (Henderson et al., 2006).

The distribution of the 21-, 22-, 23- and 24-nt reads matching the Arabidopsis genome but excluding tRNA- and rRNA-derived RNAs was examined. The two *35S::RTL2* lines and the *rtl2* mutant exhibited a small RNA profile comparable to that of the wild-type control. The 23-24-nt reads represented 90% of the small RNA reads (Figure 1A), and the distribution of unique siRNA reads along the five chromosomes was similar to that of wild-type plants (Figure 1B), suggesting that *RTL2* has no major contribution to the small RNA repertoire. By contrast, the small RNA size distribution in *35S::RTL1* plants resembled that in the *dcl2 dcl3 dcl4* triple mutant, i.e. they accumulated equal amounts of 21-22-nt and 23-24-nt reads (Figure 1A). To determine better the quantitative changes occurring in *35S::RTL1* plants, RNA gel blot analyses were performed using representative miRNA, ta-siRNA and PolIV/PolV-siRNA probes. Analysis of the *35S::RTL1* plant used for sequencing (Figure 2A), or two additional independent *35S::RTL1* transformants (Figure 2B), showed that conserved miRNA levels were largely unchanged in *35S::RTL1* plants, whereas the levels of ta-siRNAs and PolIV/PolV-siRNAs were reduced strongly.

Because canonical miRNAs accumulate to similar levels in *35S::RTL1* and wild-type plants, the sequence data were normalized to the 20 conserved, DCL1-dependent miRNAs. Analysis of the normalized chromosomal distribution of unique small RNA reads that do not correspond to conserved miRNAs, i.e. primarily siRNAs, revealed that *35S::RTL1* plants almost entirely lack siRNAs (Figure 1B). Furthermore, sorting siRNAs into their major functional categories showed that *RTL1* over-expression strongly impacts the accumulation of ta-siRNAs, endoIR-siRNAs and PolIV/PolV-siRNAs (Figure 3 for whole-genome analysis and supplemental Figures 1, 2 and 3 for the analysis of representative loci). We also analyzed the levels of young miRNAs because their long dsRNA precursors resemble more the precursors of endoIR-siRNAs than the precursors of canonical miRNAs. Similar to ta-siRNAs, endoIR-siRNAs and PolIV/PolV-siRNAs, levels of the young miRNAs miR822, miR833, miR838 and miR869 were reduced strongly in *35S::RTL1* plants (Figure 2 and supplemental Figure 4), indicating that *RTL1* affects the accumulation of all classes of endogenous small RNAs, except canonical miRNAs.



**Figure 2: RNA gel blot analysis of diverse types of small RNAs.**

Gel blots of total RNA from wild-type (Col) and 35S::*RTL1* plants were hybridized with the indicated probes. **A)** RNA blot analysis of the 35S::*RTL1*#1 plant used for sequencing in Figure 1. **B)** RNA blot analysis of two additional independent 35S::*RTL1* plants

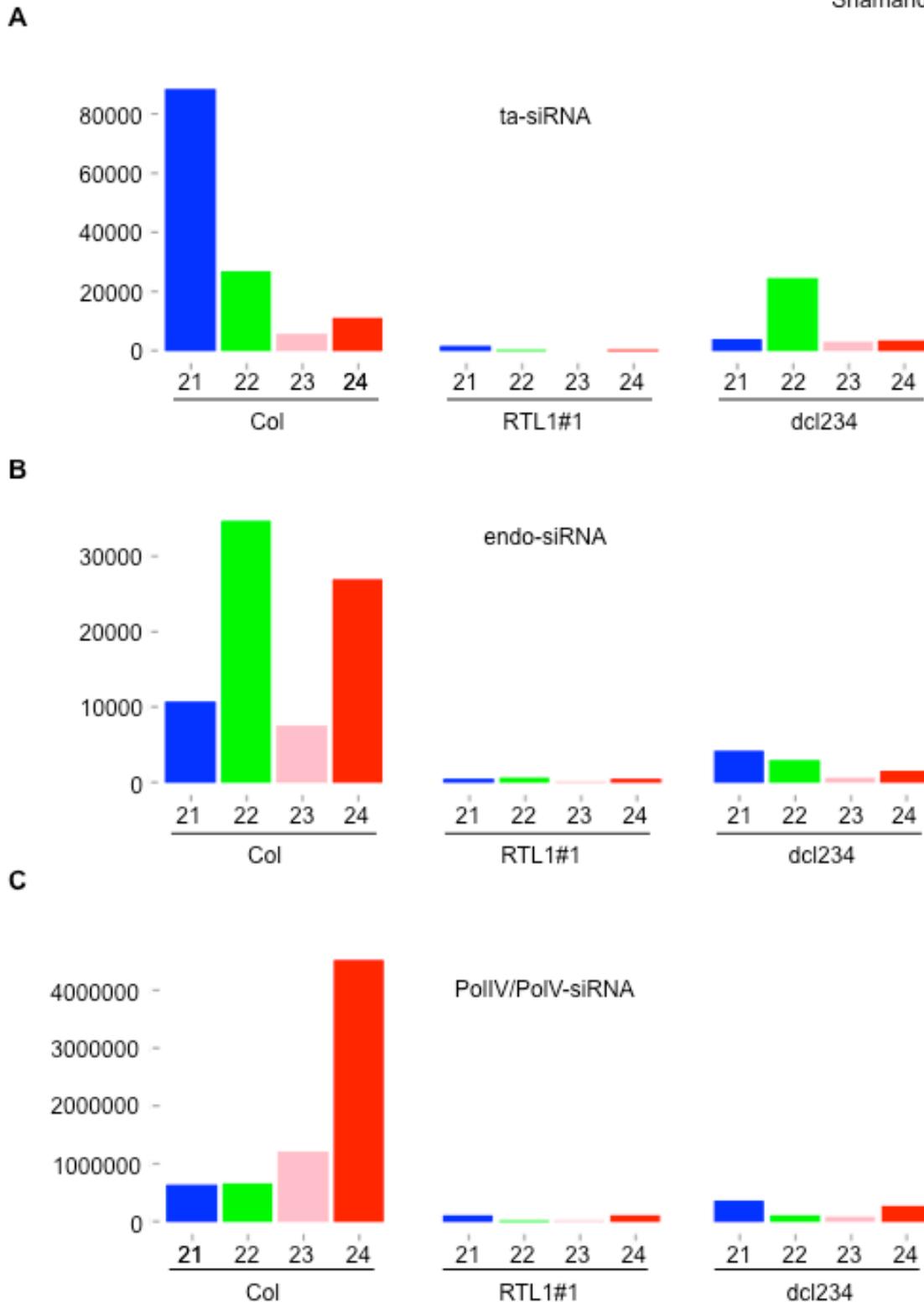
### *RTL1 inhibits transgene PTGS*

Given the ability of RTL1 to inhibit the accumulation of endogenous siRNAs, we examined whether RTL1 over-expression also impacted transgene-derived siRNAs. To this end, a *35S::GU-UG* transgene expressing an inverted repeat hairpin RNA consisting of the 5' part of the *GUS* reporter sequence (*GU*) was introduced transiently into *Nicotiana benthamiana* leaves together with either *35S::RTL1* or a *35S::GFP* control. As expected, *35S::GU-UG* + *35S::GFP* infiltrated leaves accumulated high levels of 21- and 24-nt *GU* siRNAs, whereas *35S::GU-UG* + *35S::RTL1* infiltrated leaves did not accumulate detectable levels of *GU* siRNAs (Figure 4A), indicating that RTL1 prevents the accumulation of transgene siRNAs deriving from an inverted repeat. We also determined the effect of RTL1 on PTGS, by co-infiltrating the *35S::GU-UG* construct and a target *35S::GUS* reporter with either *35S::RTL1* or a *35S::GFP* control. Leaves infiltrated with *35S::GU-UG* + *35S::GUS* + *35S::GFP* lacked *GUS* mRNA, indicating that PTGS of *GUS* was established, whereas *35S::GU-UG* + *35S::GUS* + *35S::RTL1* infiltrated leaves accumulated *GUS* mRNA (Figure 4B), indicating that RTL1 prevents transgene siRNA-mediated PTGS of the *35S::GUS* target. RTL1-mediated inhibition of transgene PTGS was confirmed in the Arabidopsis line *LI*, which undergoes spontaneous PTGS of a stably integrated *35S::GUS* reporter transgene. Transgenic *LI/35S::RTL1* plants lacked *GUS* siRNAs, whereas *LI* controls accumulated *GUS* siRNAs (Figure 4C).

### *RTL1 activity requires functional RNaseIII and DRB domains*

To determine if an intact RNaseIII domain is required for RTL1 to inhibit siRNA accumulation and PTGS, first, the conserved catalytic site amino-acids E89 and D96 of RTL1 were both mutagenized to alanine (we refer to this transgene as *RTL1mR3*). Then, *N. benthamiana* leaves were co-infiltrated with *35S::GU-UG* and either *35S::RTL1* or *35S::RTL1mR3*. *35S::GU-UG* + *35S::RTL1mR3* infiltrated leaves accumulated high levels of 21- and 24-nt *GU* siRNAs, similar to *35S::GU-UG* + *35S::GFP* infiltrated leaves, whereas *35S::GU-UG* + *35S::RTL1* infiltrated leaves lacked *GU* siRNAs (Figure 4A), indicating that the wild-type RNaseIII domain of RTL1 is required for RTL1 to impact siRNA accumulation. Next, to determine the impact on PTGS, *N. benthamiana* leaves were co-infiltrated with *35S::GU-UG* + *35S::GUS* and either *35S::RTL1* or *35S::RTL1mR3*. *35S::GU-UG* + *35S::GUS* + *35S::RTL1mR3* infiltrated leaves silenced *GUS* and accumulated *GUS* siRNAs and lacked *GUS* mRNA, similar to *35S::GU-UG* + *35S::GUS* + *35S::GFP* infiltrated leaves, whereas *35S::GU-UG* + *35S::GUS* + *35S::RTL1* infiltrated leaves lacked *GUS* siRNAs and accumulated *GUS* mRNA (Figure 4B), indicating that an intact RTL1 RNaseIII domain is necessary to suppress PTGS.

To monitor RTL1 and RTL1mR3 protein accumulation, two different epitope tags were added individually to RTL1 as C-terminal or N-terminal fusions, and then the RNaseIII domain was

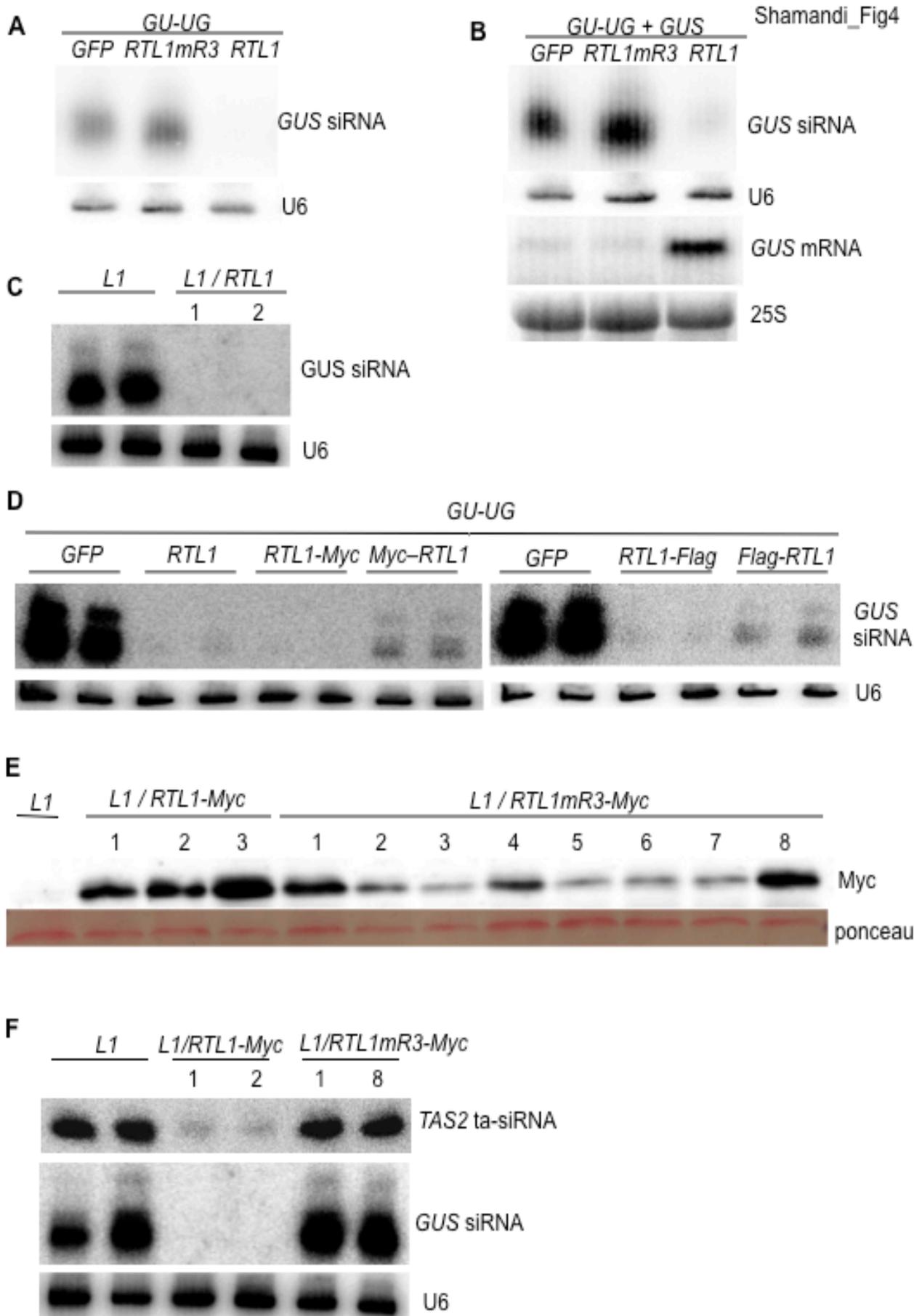


**Figure 3: Normalized abundance of the three major classes of endogenous siRNAs.**

Small RNAs from wild-type (Col), *35S::RTL1* transgenic plants and *dcl2dcl3dcl4* mutants were classified as ta-siRNAs (A), endoIR-siRNAs (B) and PolIV/PoIV-siRNAs (C) based on published annotation. Small RNA abundance was normalized to the total amount of conserved miRNAs. Each size of small RNA is indicated by a different color: 21-nt (blue), 22-nt (green), 23-nt (pink) and 24-nt (red).

mutagenized. To determine if the presence of the epitope tags impaired wild-type RTL1 function, the *35S::GU-UG* construct was infiltrated into *N. benthamiana* leaves with either *35S::RTL1*, *35S::Flag-RTL1*, *35S::RTL1-Flag*, *35S::Myc-RTL1* or *35S::RTL1-Myc* constructs. The *35S::RTL1-Flag* and *35S::RTL1-Myc* constructs reduced *GU* siRNA accumulation almost as efficiently as the *35S::RTL1* control, whereas the *35S::Flag-RTL1* and *35S::Myc-RTL1* constructs had a weaker effect (Figure 4D), suggesting that the addition of these N-terminal tags impacted RTL1 activity. Consistent with this observation, *35S::Flag-RTL1* and *35S::Myc-RTL1* Arabidopsis transformants did not exhibit developmental defects, whereas *35S::RTL1-Flag* and *35S::RTL1-Myc* transformants displayed developmental defects similar to, although less severe than, *35S::RTL1* plants (supplemental Figure 5A). Indeed, similar to *35S::RTL1* plants, both *35S::RTL1-Flag* and *35S::RTL1-Myc* plants exhibited downward leaf curling, typical of plants lacking TAS3 ta-siRNAs. However, they were less dwarf, yellow and sterile than *35S::RTL1* plants (data not shown). The *35S::RTL1-Flag* and *35S::RTL1-Myc* constructs were retained for further analysis because, among the four tagged constructs, they showed the strongest reduction in siRNA accumulation (supplemental Figure 5B), and the strongest effect on *L1* PTGS (supplemental Figure 5C).

To ensure that the absence of developmental and molecular defects in *35S::RTL1mR3* plants was not due to destabilization or impaired production of the RTL1 protein, the RNaseIII domain of the *35S::RTL1-Myc* construct was mutagenized as previously described, and *N. benthamiana* leaves were co-infiltrated with the *35S::GU-UG* and either *35S::GFP*, *35S::RTL1-Myc* or *35S::RTL1mR3-Myc* constructs. *35S::GU-UG* + *35S::RTL1mR3-Myc* infiltrated leaves accumulated high levels of 21- and 24-nt *GU* siRNAs, whereas *35S::GU-UG* + *35S::RTL1-Myc* infiltrated leaves lacked *GU* siRNAs (Figure 4D and supplemental Figure 6A), similar to the results obtained with non-tagged *RTL1* and *RTL1mR3* constructs (Figure 4A). However, western blot analysis revealed that the RTL1mR3-Myc protein accumulated to a lower level than the RTL1-Myc protein in infiltrated leaves (supplemental Figure 6A), raising concerns about the effect of the two introduced mutations on protein stability. To address this question, Arabidopsis *L1* plants were transformed with either *35S::RTL1-Myc* or *35S::RTL1mR3-Myc*, and the amount of RTL1-Myc and RTL1mR3-Myc protein was determined by western blot in a series of individual transformed plants (Figure 4E). Two *L1/35S::RTL1-Myc* (#1 and #2) and two *L1/35S::RTL1mR3-Myc* (#1 and #8) plants accumulating comparable amounts of RTL1 protein were selected for analysis of endogenous and transgene siRNAs. Both *L1/35S::RTL1-Myc* plants accumulated *GUS* mRNA and lacked *GUS* siRNAs and endogenous ta-siRNAs, whereas *L1/35S::RTL1mR3-Myc* plants lacked *GUS* mRNA and accumulated *GUS* siRNAs and endogenous ta-siRNAs (Figure 4F and data not shown), indicating that the wild-type RTL1 RNaseIII domain is required to suppress small RNA accumulation.



**Figure 4: The RTL1 RNaseIII domain is required for inhibition of transgene PTGS**

A) *Nicotiana benthamiana* leaves were infiltrated with a 35S::*GU-UG* construct (*GU-UG*) together with either a wild-type 35S::*RTL1* construct (*RTL1*), a construct mutated in the RNaseIII domain (*RTL1mR3*) or a 35S::*GFP* control (*GFP*). Low molecular weight (LMW) RNAs were hybridized with a *GUS* probe and with *U6* as a loading control. B) *Nicotiana benthamiana* leaves were infiltrated with a 35S::*GU-UG* construct (*GU-UG*) and a 35S::*GUS* construct (*GUS*) together with either a wild-type 35S::*RTL1* construct (*RTL1*), a construct mutated in the RNaseIII domain (*RTL1mR3*) or a 35S::*GFP* control (*GFP*). LMW RNAs were hybridized with a *GUS* probe and with *U6* as loading control. High molecular weight RNAs were hybridized with a *GUS* probe and with 25S as loading control. C) The Arabidopsis line *LI* carrying a 35S::*GUS* transgene silenced by PTGS was transformed with a wild-type 35S::*RTL1* construct (*RTL1*). LMW *GUS* RNA from two independent transformants were analyzed. D) *Nicotiana benthamiana* leaves were infiltrated with a 35S::*GU-UG* construct (*GU-UG*) together with either a wild-type 35S::*RTL1* construct (*RTL1*), tagged constructs (*RTL1-Myc*, *Myc-RTL1*, *RTL1-Flag*, *Flag-RTL1*) or a 35S::*GFP* control (*GFP*). LMW RNAs were hybridized with a *GUS* probe and with *U6* as a loading control. E) The Arabidopsis line *LI* was transformed with either a wild-type tagged 35S::*RTL1* construct (*RTL1-Myc*) or a tagged construct mutated in the RNaseIII domain (*RTL1mR3-Myc*). Proteins were extracted from three independent *RTL1-Myc* transformants and eight independent *RTL1mR3-Myc* transformants and hybridized with a Myc antibody. F) LMW RNAs from *RTL1-Myc* and *RTL1mR3-Myc* transformants expressing comparable amount of proteins were hybridized with *GUS* and *TAS2* probes and with *U6* as a loading control.

To determine if RTL1 activity also requires its DRB domain, a *35S::RTL1-ΔDRB-Myc* construct lacking the C-terminal DRB domain was generated. *35S::GU-UG* + *35S::RTL1-ΔDRB-Myc* infiltrated *N. benthamiana* leaves accumulated high levels of 21- and 24-nt *GU* siRNAs, similar to *35S::GU-UG* + *35S::GFP* infiltrated leaves, indicating that the DRB domain is required for RTL1 activity (supplemental Figure 6B).

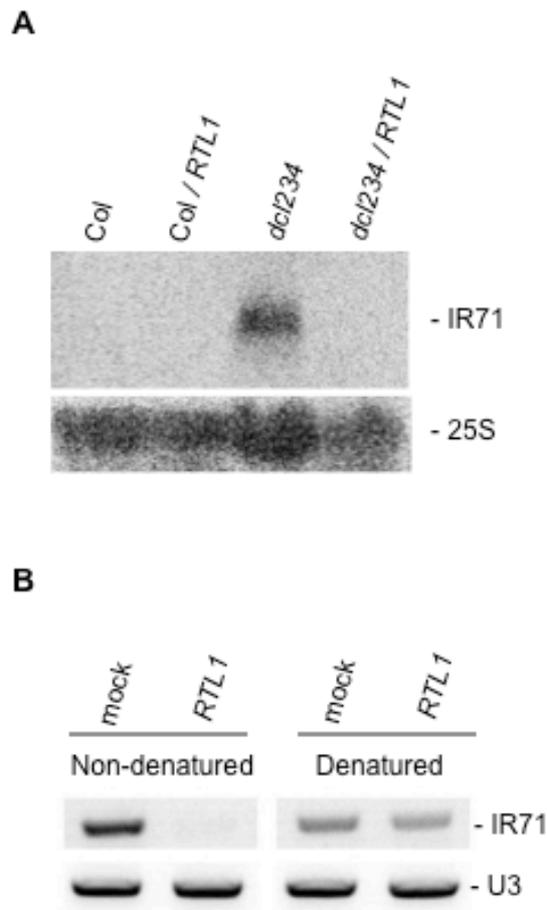
#### *RTL1 cleaves near-perfectly paired long dsRNA*

The selective effect of RTL1 on young miRNAs, ta-siRNAs, endoIR-siRNAs, p4/p5-siRNAs and transgene siRNAs could be explained by several, non-exclusive mechanisms:

- 1) inhibition of the enzymes involved in the biogenesis of their dsRNA precursors,
- 2) degradation of their dsRNA precursors,
- 3) inhibition of or competition with the DCLs processing their dsRNA precursors
- 4) degradation of these small RNAs.

The fact that RTL1 over-expression impairs the accumulation of young miRNAs and endoIR-siRNAs rules out that RTL1 inhibits RDR activity. Indeed, young miRNAs and endoIR-siRNAs are processed from self-complementary single-stranded RNAs that do not require RDR for their production. The fact that levels of young miRNAs, ta-siRNAs, endoIR-siRNAs and p4/p5-siRNAs are more strongly reduced in *35S::RTL1* plants than in the *dcl2 dcl3 dcl4* triple mutant (Figure 1B and Figure 3) makes also unlikely that RTL1 inhibits DCL2, DCL3 and DCL4 activity or that RTL1 selectively degrades small RNAs produced by DCL2, DCL3 and DCL4. Indeed, the residual level of siRNAs observed in the *dcl2 dcl3 dcl4* triple mutant likely results from a weak activity of DCL1 on siRNA precursors in the absence of DCL2, DCL3 and DCL4, and the disappearance of these siRNAs in *35S::RTL1* plants suggests that RTL1 acts upstream of the DCLs. Therefore, it is very tempting to speculate that RTL1 degrades the dsRNA precursors of young miRNAs, ta-siRNAs, endoIR-siRNAs, p4/p5-siRNAs and transgene siRNAs, thereby preventing their processing by any of the four DCLs. To test this hypothesis, we examined the accumulation of the endoIR-siRNA precursor *IR71* because *IR71*-derived siRNAs are totally absent in plants expressing the *35S::RTL1* construct (Supplemental Figure 2). As shown before (Dunoyer et al, 2010), the *IR71* precursor accumulates in the *dcl2 dcl3 dcl4* triple mutant, but does not accumulate in wild-type Col, likely because it is efficiently processed by DCL2, DCL3 and DCL4 (Figure 5A). The *IR71* precursor did not accumulate in the *dcl2 dcl3 dcl4* triple mutant transformed with the *35S::RTL1* construct (Figure 5A), suggesting that it is degraded/destabilized by RTL1.

Confirmation that RTL1 acts on dsRNA was further obtained using an *in vitro* cleavage assay. A soluble His-RTL1 fusion protein was expressed in *E. coli*, purified to near homogeneity and incubated



**Figure 5: RTL1 cleaves dsRNA.**

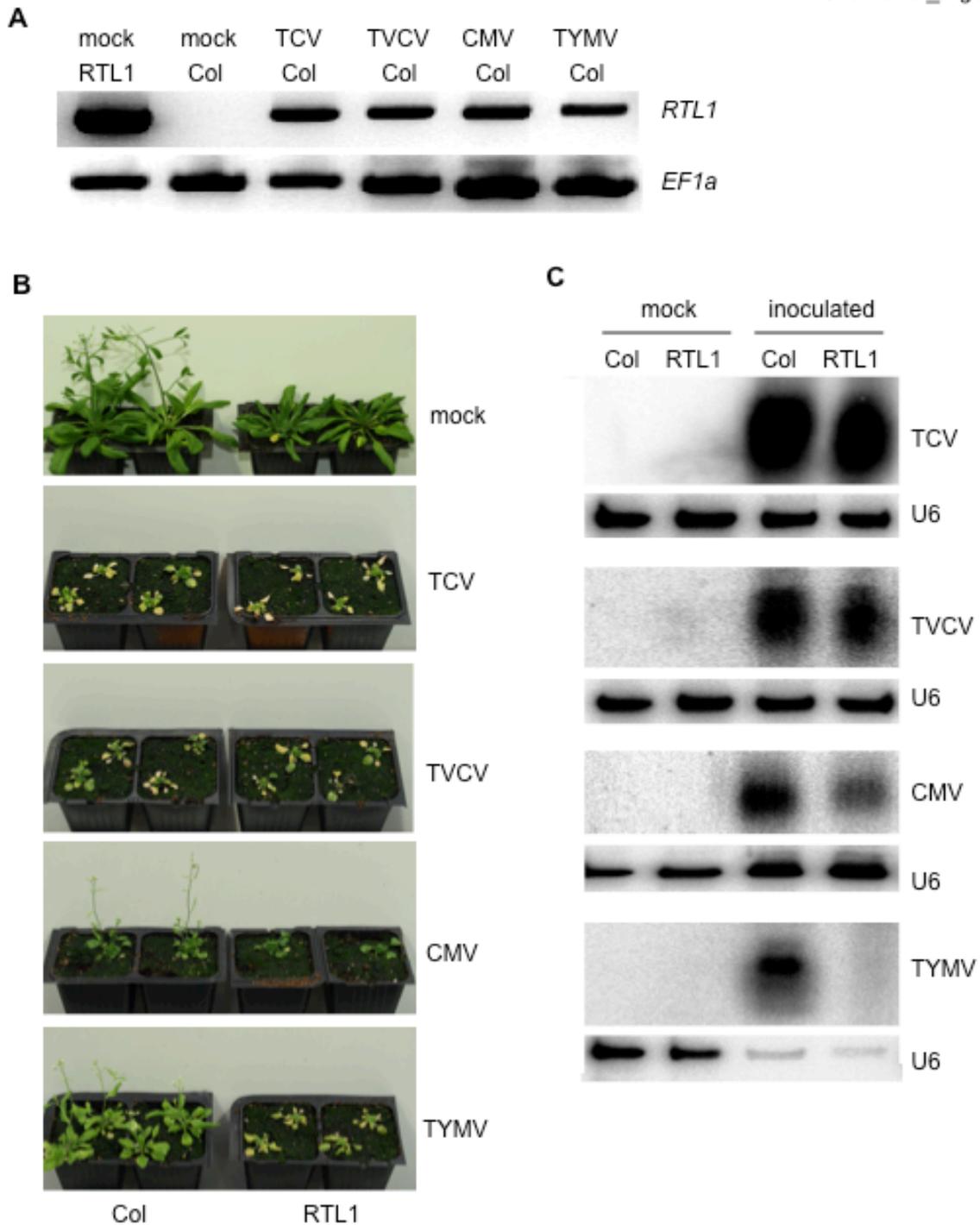
A) RNA gel blot detection of *IR71* precursor RNA in wild-type (Col), Col transformed with the *35S::RTL1* construct (*Col/RTL1*), the *dcl2dcl3dcl4* triple mutant (*dcl234*), and *dcl234* transformed with the *35S::RTL1* construct (*dcl234/RTL1*). Transformants exhibiting a strong RTL1 developmental phenotype were analyzed. High molecular weight (HMW) RNAs were hybridized with a probe complementary to the *IR71* RNA and with *25S* as loading control. B) RT-PCR reactions to detect *IR71* precursor RNA and *U3* snoRNA in Arabidopsis RNA samples. RNAs were denatured or not before incubation with the His-AtRTL1 recombinant protein (RTL1). Untreated samples were used as controls (mock). RT-PCR amplification of *U3* snoRNA sequences shows similar amount of RNA in each reaction.

with total RNA extracted from wild-type *Arabidopsis* seedlings. RT-PCR analysis reveals that purified His-RTL1 cleaves *IR71* precursor RNA, which can be visualized by the absence of amplification in the presence of His-RTL1, whereas amplification is detected in RNA fractions incubated without His-RTL1 (Figure 5B). The specificity of RTL1 towards dsRNA was demonstrated by incubating His-RTL1 with denatured or non-denatured RNA. Indeed, His-RTL1 was able to cleave non-denatured but not denatured *IR71* precursor RNA (Figure 5B). However, His-RTL1 activity is specific to certain dsRNA. Indeed, the abundant and highly structured *U3* snoRNA (Marshall et al., 1990), which is not a siRNA precursor, was not cleaved by His-RTL1 (Figure 5B), indicating that RTL1 cleavage activity is sequence- or structure-specific, or depends on a co-factor that recognizes siRNA precursors.

#### *Viruses induce RTL1 mRNA but viral suppressors of RNA silencing counteract RTL1 activity*

Given its ability to cleave dsRNA, RTL1 could play several non-exclusive roles in plant-virus interactions. Indeed, RTL1 could cleave dsRNA intermediates of virus replication generated by viral RNA-dependent RNA polymerase (RdRP), which in theory should enhance plant resistance. However, RTL1 could also cleave viral dsRNAs generated by cellular RNA-dependent RNA polymerase (RDR), which in theory should enhance plant susceptibility because RDR-derived dsRNAs give rise to siRNAs that normally are loaded onto AGO1 and AGO2, which target viral RNAs for destruction. However, RTL1 is not expressed under normal laboratory conditions in leaves, and is only weakly expressed in roots (Comella et al, 2008). Thus, RTL1 likely could only play a role in plant-virus interactions if it is induced during virus infection. To test this hypothesis, we inoculated wild-type Col plants with representative viruses from four different families: *turnip crinkle virus* (TCV), *turnip vein clearing virus* (TVCV), *cucumber mosaic virus* (CMV) and *turnip yellow mosaic virus* (TYMV). Upon inoculation, *RTL1* mRNA accumulated in systemic leaves whereas it was below detectable levels in mock-inoculated plants (Figure 6A), indicating that *RTL1* expression is generally induced in response to virus infection.

To further examine the biological role of RTL1 in plant-virus interactions, we attempted to compare virus-sensitivity in wild-type Col and *rtl1* mutants. However, *rtl1* null alleles could not be found in publicly available libraries. Moreover, neither of two artificial miRNAs directed against RTL1 (amiR-RTL1a and amiR-RTL1b) was able to suppress the RTL1 overexpression phenotype conferred by the *35S::RTL1* construct, despite an abundant accumulation of the two amiRNAs (Supplemental Figure 7), suggesting that *RTL1* mRNA is reluctant to silencing. Therefore, we attempted to address the biological role of RTL1 in plant-virus interactions by comparing the responses of wild-type Col and *35S::RTL1-Flag* plants after infection with the four representative viruses used in our previous experiments. For this purpose, we chose a *35S::RTL1-Flag* line that accumulates *RTL1* mRNA at a



**Figure 6: RTL1 impact on virus infection.**

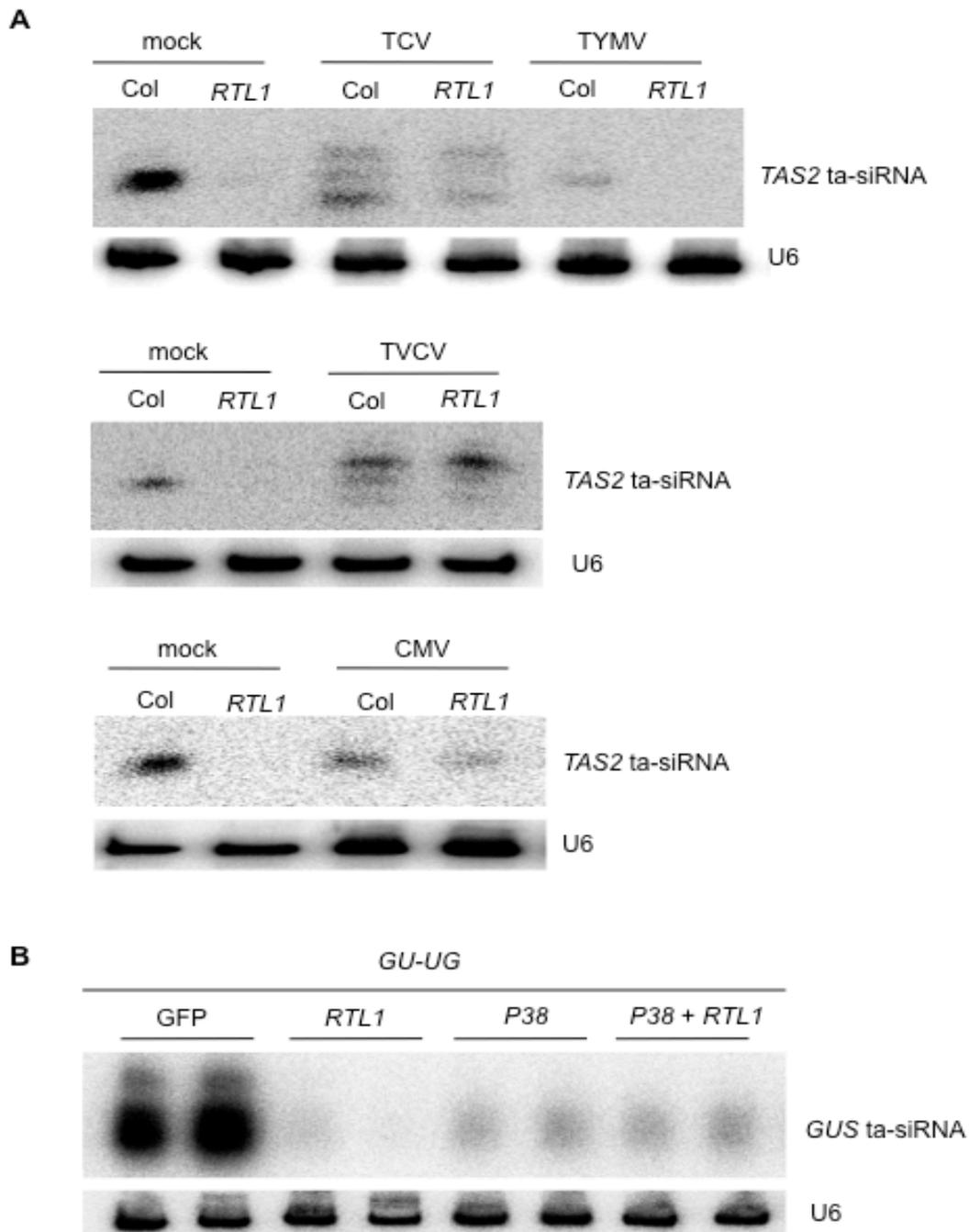
A) RNAs extracted from mock-inoculated wild-type (Col) and *35S::RTL1-Flag* plants (*RTL1*) and from TCV-, TVCV-, CMV- and TYMV-inoculated wild-type (Col) were subjected to oligo-dT reverse transcription followed by PCR with oligos specific to either *RTL1* or *EF1a*. B) Pictures of mock-, TCV-, TVCV-, CMV- and TYMV-inoculated wild-type (Col) and *35S::RTL1-Flag* plants (*RTL1*). Ten-day-old plants were inoculated. Pictures were taken three weeks following inoculation. C) RNA gel blot detection of TCV, TVCV, CMV and TYMV siRNAs in mock- and virus-inoculated wild-type (Col) and *35S::RTL1-Flag* plants (*RTL1*). *U6* was used as a loading control.

higher level than that observed in wild-type Col inoculated with viruses so that the effect of *RTL1* over-expression could be determined.

*RTL1* over-expression caused a severe aggravation of TYMV-induced symptoms. Indeed, whereas TYMV-inoculated wild-type Col plants only showed mild symptoms and were able to bolt, flower and set seeds, TYMV-inoculated *35S::RTL1-Flag* plants resembled TCV- and TVCV- inoculated wild-type plants, which are unable to bolt and which eventually die from the infection (Figure 6B). TYMV-derived siRNAs accumulated in TYMV-inoculated wild-type Col plants, but they were below detectable level in TYMV-inoculated *35S::RTL1-Flag* plants (Figure 6C). These results indicate that, when artificially over-expressed, RTL1 prevents the accumulation of TYMV siRNAs as efficiently as it prevents the accumulation of transgene siRNAs (Figure 4), thus making RTL1 an antagonist of plant antiviral PTGS defenses. Thus, *35S::RTL1-Flag* plants are deprived of the plant antiviral PTGS defense, which normally limits the proliferation of TYMV by targeting TYMV RNA for destruction, resulting in severe symptoms of viral infection.

In contrast to TYMV, TCV-, TVCV- and CMV-inoculated *35S::RTL1-Flag* plants did not show aggravated viral symptoms compared with wild-type Col infected plants (Figure 6B). Indeed, TCV- and TVCV-inoculated wild-type Col plants developed severe symptoms similar to TCV- and TVCV-inoculated *35S::RTL1-Flag* plants, and all these plants eventually died from the infection. CMV-inoculated *35S::RTL1-Flag* plants developed milder symptoms than TCV- and TVCV-inoculated *35S::RTL1-Flag* plants; however, these symptoms were similar to that of CMV-inoculated wild-type plants (note that the late flowering phenotype observed in CMV-inoculated *35S::RTL1-Flag* plants is also observed in mock-inoculated *35S::RTL1-Flag* plants). Strikingly, TCV- and TVCV-derived siRNAs accumulated at similar levels in wild-type and *35S::RTL1-Flag* plants (Figure 6C), as if RTL1 was unable to prevent the accumulation of TCV- and TVCV-derived siRNAs. CMV-derived siRNAs also accumulated in *35S::RTL1-Flag* plants, although at a level lower than in wild-type Col plants (Figure 6C), suggesting that RTL1 only partially prevents the accumulation of CMV-derived siRNAs.

These results indicate that viruses induce the expression of the native RTL1 gene, but that most of them have the capacity to inhibit the anti-silencing activity of RTL1, either completely (TCV, TVCV) or partially (CMV). To test this hypothesis, the accumulation of endogenous ta-siRNAs was compared in TCV-, TVCV-, CMV- and TYMV-inoculated *35S::RTL1-Flag* and wild-type Col plants. *TAS2* ta-siRNAs of the correct 21nt length accumulated in both TYMV-inoculated wild-type plants and mock-inoculated wild-type plants, although at a lower level TYMV-inoculated plants (Figure 7A). However, they were absent in both TYMV-inoculated *35S::RTL1-Flag* plants and mock-inoculated *35S::RTL1-*



**Figure 7: Virus impact on RTL1 activity.**

A) RNAs extracted from mock- or virus inoculated wild-type (Col) and *35S::RTL1-Flag* plants (*RTL1*) were hybridized with a *TAS2* probe and *U6* as a loading control. B) *Nicotiana benthamiana* leaves were infiltrated with a *35S::GU-UG* construct (*GU-UG*) together with either a wild-type *35S::RTL1* construct (*RTL1*), a *35S::P38* construct (*P38*), a mix of the *35S::RTL1* and *35S::P38* constructs (*P38 + RTL1*), or a *35S::GFP* control (*GFP*). LMW RNAs were hybridized with a *GUS* probe and with *U6* as a loading control.

*Flag* plants, confirming that TYMV does not inhibit RTL1 activity. In contrast, *TAS2* ta-siRNAs accumulated in TCV-, TVCV- and CMV-inoculated *35S::RTL1-Flag* plants, whereas they were absent in mock-inoculated *35S::RTL1-Flag* plants (Figure 7A), confirming that TCV, TVCV and CMV reduce RTL1 activity. Note that the difference in ta-siRNA size between mock-inoculated and TCV-inoculated wild-type plants results from the inhibiting effect of the TCV P38 protein on DCL4 activity (Bouche et al., 2006; Deleris et al., 2006; Schott et al., 2012).

Together, these results indicate that RTL1 expression is induced in response to virus infection, but that some viruses have evolved strategies to inhibit RTL1 activity. It is long known that most viruses encode proteins that inhibit components of the plant antiviral PTGS defense, which allow most viruses to counteract plant defenses and establish infections. These proteins are referred to as viral suppressors of RNA silencing (VSR). To determine if VSR proteins are also responsible for the inhibition of RTL1 activity, the well-characterized TCV VSR P38 was used in Agro-infiltration experiments. *N. benthamiana* leaves were co-infiltrated with the *35S::GU-UG* construct and either *35S::GFP*, *35S::RTL1*, *35S::P38* constructs, or *35S::RTL1 + 35S::P38* constructs. Whereas *35S::RTL1* infiltrated leaves exhibited strongly reduced levels of *GU* siRNAs compared with *35S::GFP* infiltrated leaves, *35S::RTL1 + 35S::P38* infiltrated leaves accumulated *GU* siRNAs at a level similar to that of *35S::P38* infiltrated leaves (Figure 7B). These results indicate that P38 is sufficient to prevent RTL1-mediated inhibition of transgene-derived siRNAs, and suggest that P38 likely also prevents RTL1-mediated inhibition of TCV-derived siRNAs (Figure 6C).

## Discussion

Unlike the DICER-type RNaseIII enzymes that produce small RNAs necessary for silencing in a range of eukaryotes, the plant non-DICER RNaseIII enzyme RTL1 does not promote small RNA production but rather represses it. Genome-wide profiling of small RNAs in plants ectopically expressing RTL1 under the control of a strong promoter (*35S::RTL1*) revealed that RTL1 inhibits the accumulation of all types of small RNAs normally produced by DCL2, DCL3 or DCL4. Because RTL1 over-expression does not affect the accumulation of DCL1-dependent conserved miRNAs, which are processed from imperfectly-paired short hairpins, it is likely that RTL1 specifically affects perfectly paired long dsRNA, such as the precursors of ta-siRNAs and p4/p5-siRNAs, which derive from RDR activity, or near-perfectly paired long dsRNA, such as the precursors of young miRNAs and of endoIR-siRNAs. Consistently, *in vivo* and *in vitro* assays show that RTL1 cleaves dsRNA. Because siRNAs are almost totally absent in *35S::RTL1* plants where the four DCLs are present, RTL1 likely has a very high affinity for long dsRNA and thus cleaves dsRNA before they enter processing by the DCLs.

RTL1 is conserved in plants (Supplemental Figure 8), suggesting that it has essential functions. When overexpressed, not only does RTL1 interfere with the accumulation of endogenous siRNAs, but it also interferes with the accumulation of exogenous siRNAs, such as transgene-derived siRNAs, indicating that RTL1 is a general suppressor of plant siRNA pathways. However, under laboratory growth conditions, RTL1 is very weakly expressed in the roots and below detection levels in leaves and flowers (Comella et al., 2008), consistent with the accumulation of siRNAs in these tissues in wild-type plants. RTL1 is induced in leaves in response to viral infection, suggesting that upon viral infection, RTL1 could suppress the accumulation of virus-derived siRNAs and thus counteract the plant anti-viral PTGS defense. PTGS was first identified in plants that did not express transgenes, but it rapidly became obvious that it naturally serves as a mechanism of antiviral defense in plants, flies and worms (Ding and Voinnet, 2007; Felix et al., 2011; Li et al., 2002; Mourrain et al., 2000). Indeed, a fraction of viral RNA is transformed into siRNAs by Dicer enzymes (DCL2 and DCL4 in plants), which, upon loading onto AGO proteins (AGO1 and AGO2 in plants), target viral RNA for destruction (Wang et al., 2011). However, this mechanism is rarely efficient to protect plants against infection because most viruses have evolved proteins that suppress PTGS at various steps (Ding and Voinnet, 2007). These proteins are referred to as viral suppressors of RNA silencing (VSR). For example, CMV encodes 2b, a VSR protein that exhibits the dual activity of binding dsRNA and AGO1, thus blocking its activity (Duan et al., 2012). TCV encodes P38, a VSR protein that also binds AGO1 and blocks its activity. In addition, P38 indirectly alters the activity of DCL4 (Azevedo et al., 2010; Deleris et al., 2006b; Schott et al., 2012; Zhang et al., 2006). Besides targeting cellular components of the PTGS machinery, viruses can also suppress plant PTGS by expressing VSR proteins that target RNAs. For example, TBSV encodes P19, a VSR that sequesters siRNAs (Silhavy et al., 2002), while SPCSV encodes an RNaseIII enzyme that likely degrades siRNAs (Cuellar et al., 2009). As a result of the action of VSRs, viruses generally succeed to infect plants. However, each VSR exhibits a different strength in blocking plant antiviral PTGS defenses. For example, the VSR activity of TYMV P69 is weaker than that of CMV 2b, which is weaker than that of TCV P38, resulting in mild symptoms in TYMV-infected Arabidopsis, intermediate symptoms in CMV-infected Arabidopsis and severe symptoms in TCV-infected Arabidopsis, as well as partial suppression of transgene PTGS by TYMV (Chen et al., 2004), intermediate suppression by CMV and complete PTGS suppression by TCV (Beclin et al., 1998; Mourrain et al., 2000).

Surprisingly, plants also encode proteins that counteract their antiviral PTGS defense. For example, XRN4 encodes a 5'→3' exoribonuclease that likely degrades viral substrates of PTGS. Indeed, *xrn4* mutants exhibit hyper-resistance to virus infection, indicating that XRN4 counteracts PTGS in wild-type plants (Gy et al., 2007). Here, we show that RTL1 has the potential to counteract the plant antiviral PTGS defense. Indeed, *35S::RTL1* plants exhibit hyper-susceptibility to infection by TYMV and lack TYMV-derived siRNAs, whereas wild-type plants exhibit mild symptoms and produce

siRNAs that target TYMV RNAs for destruction by PTGS. However, other viruses, such as TCV, TVCV or CMV cause similar symptoms on wild-type and *35S::RTL1* plants. Moreover, unlike TYMV-infected *35S::RTL1* plants that lack virus-derived siRNAs and endogenous siRNAs, TCV-, TVCV- and CMV-infected *35S::RTL1* plants accumulate virus-derived siRNAs and endogenous siRNAs, suggesting that RTL1 is unable to prevent the accumulation of viral siRNAs because its activity is inhibited by these viruses. If so, it is unlikely that RTL1 contributes to the successful infection of wild-type and *35S::RTL1* plants by TCV, TVCV and CMV. Rather, it is likely that successful infection by these viruses relies on the inhibition of the plant antiviral PTGS defense by these viruses, consistent with TCV, TVCV and CMV strongly suppressing transgene PTGS (Beclin et al., 1998; Mourrain et al., 2000), whereas TYMV only weakly suppresses transgene PTGS (Chen et al., 2004).

Since RTL1 has the potential to counteract the plant antiviral PTGS defense by cleaving dsRNA precursors of viral siRNAs and preventing the loading of virus-derived siRNAs onto antiviral AGO proteins, why do TCV, TVCV and CMV inhibit RTL1 activity? Indeed, the induction of RTL1 expression, which is generally observed upon virus infection, should only favor virus proliferation, as observed in the case of TYMV. The fact that TCV, TVCV and CMV strongly inhibit RTL1 activity suggests that the inhibition of RTL1 by certain viruses is essential for other reasons. For example, it may be necessary for certain viruses to inhibit RTL1 activity if RTL1 cleaves their dsRNA replication intermediates, which would impair infection early on. Thus, it is reasonable to assume that viruses that are sensitive to RTL1 at some step(s) of their replication have evolved strategies to inhibit or counteract RTL1 activity. Interestingly, inhibition of antiviral RTL1 activity appears to rely on VSR proteins that also inhibit antiviral plant PTGS defenses. Viral-mediated suppression of RTL1 also could be a strategy to prolong the life of their host plant so that a successful infection can be established before the plant succumbs to death. It will be interesting to determine if viruses also suppress the activity of other known endogenous silencing suppressors.

Our study has revealed a novel plant protein involved in plant-virus interactions. In the case of TYMV, RTL1 serves as an endogenous suppressor of the PTGS defense pathway that may help viruses to infect plants, likely by degrading dsRNA precursors of virus-derived siRNAs. However, the inhibition of RTL1 activity by TCV, TVCV and CMV suggests that RTL1 may also serve as an actor of the cellular defenses that help plants to fight against viruses, for example by degrading dsRNA intermediates of virus replication. Further experiments will be required to address the precise action of RTL1 towards each virus.

## Materials and Methods

### *Plant material, transformation and virus inoculation*

*Arabidopsis* plants are in the Col-0 ecotype. The mutants *rtl2* and *dcl2dcl3dcl4* and the line *L1* have been previously described (Comella et al., 2008; Elmayan et al., 1998; Henderson et al., 2006).

Plants were grown in standard long-day conditions (16h of light and 8 h of dark at 20°C).

*Arabidopsis* was transformed using the floral-dip method.

For virus infection, 10-day-old in vitro plants were inoculated with TVC, TVCV, CMV and TYMV 1 day after being transferred to soil. Virus inoculations were performed as previously described (Bouche et al., 2006; Mourrain et al., 2000). Plants were grown for 3 weeks under standard long-day conditions (14h of light and 10 h of dark at 20°C), after which the aerial parts of 4 to 12 plants were harvested.

For *Nicotiana benthamiana* agroinfiltrations, 10 ml cultures of *Agrobacterium tumefaciens* C58C1 (pMP90) carrying the plasmids of interest were grown overnight at 28°C and then centrifuged at 6000 rpm. The bacterial pellets were suspended at 10mM MgCl<sub>2</sub>, 10mM MES pH5.2, 150 µM acetosyringone solution to a final OD<sub>600</sub> of 1.0. The resuspended bacterial solution was incubated at 20°C for 3 hours and then used to infiltrate leaves. Leaves were harvested 3 days after infiltration.

### *Plant DNA, RNA and protein analyses*

*RTL1* (*At4g15417*) and *RTL2* (*At3g20420*) genomic sequences starting at the ATG and ending at the stop codon were PCR-amplified and cloned into the *pDONR207* (Gateway® Technology- Invitrogen) using *attB1RTL1F*, *attB2RTL1R*, *attB1RTL2F* and *attB2RTL2R* Gateway adapted oligonucleotides (supplemental Table 1). LR reactions were performed to create the *RTL1* N- or C-terminal *Flag* and *Myc* fusions under the control of the *35S* promoter of the *pGWB* Gateway series. Point mutations in the conserved amino acids of the *RTL1* RNaseIII domain were done using QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The G89 and D96 amino acids were replaced by an A using the *RTL1m2-F* and *RTL1m2-R* oligonucleotides (supplemental Table1). The *RTL1ADR* sequence was amplified using the *attB1RTL1F* and *attB2RTL1-R3-R* oligonucleotides (Supplement Table1), recombined into the *pDONR207* (Gateway® Technology- invitrogen) and then transferred to the *pGWB* Gateway series under the control of the *35S* promoter .

RNA extraction and hybridization were performed as previously described (Mallory et al., 2009). A phosphorimager LAS-4000 Fujifilm was used to quantify the hybridization signals. Oligonucleotides used as probes for small RNA detection are listed in Supplemental Table 1. For IR71 precursor RNA detection, a DNA probe was generated by PCR using the IR71fwd1 and IR71rev1 primers. For *RTL1* RT-PCR analysis, cDNA was synthesized using 2 µg of total RNA digested with DNaseI (Fermentas). 500 ng of final DNA-free RNA was used for a reverse transcription with oligo-dT (Fermentas). The PCR was performed using Taq DNA polymerase (Thermo Scientific-Fermentas) and 1 µl of RT

reaction using *RTL1-6* and *RTL1-7* oligonucleotides (supplemental Table 1). *EF1 $\alpha$*  was used as a standard.

Plant protein extraction and western blots were performed as previously described (Mallory et al., 2009). Signals were revealed using Amersham ECL Select™ kit following the manufacturer specifications.

#### *In vitro assays*

Cloning of a full-length cDNA sequence encoding RTL1 was performed by a reverse transcription polymerase reaction (RT-PCR) as described before (Comella et al., 2008). A cDNA fragment encoding amino acids 1 to 289 of RTL1 was cloned into the NdeI/XhoI site of the pET16b plasmid (Novagen, Madison, WI) using *5rtl1NdeI* and *3rtl1XhoI* primers to produce the His-tagged-RTL1 recombinant fusion protein.

The recombinant His-RTL1 proteins were produced following Novagen's instructions. Briefly, lysis was performed in Binding buffer supplemented with Dnase (20 ug/ul), MgCl<sub>2</sub> (10mM final concentration) and Protease inhibitor cocktail (Roche). After cell disruption and centrifugation, the soluble fraction was purified using the Ni<sup>2+</sup> resin. Following elution, purified His-RTL1 proteins were finally dialyzed against Sample Buffer (20 mM Tris, pH7,5, 100 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM DTT).

For the cleavage assay, total RNA was extracted from 14-day-old *A. thaliana* plantlets using TriZol reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After treatment with Turbo DNase (Ambion), 500 ng of RNA were premixed in reaction Buffer (20mM Tris, pH 7,5, 50 mM MgCl<sub>2</sub>). For denaturation, the RNA premix was boiled at 70°C for 5 minutes and immediately placed on ice at least 5 minutes before adding proteins. To assay RTL1 activity, 100 ng of recombinant RTL1 or Sample Buffer were added and incubated for 1 hour on ice. Finally, the reaction was stopped by heating samples to 70°C for 5 minutes. 50 ng of treated RNAs were used for reverse transcription (RT) (Promega) using the IR71rt and U3rt primers, followed by 42 cycles of PCR using IR71fwd2 and IR71rev2 primers, and U3fwd and U3rt primers, respectively, using the *Gotaq PCR Kit* (Promega).

#### *Bioinformatics*

The 3' adapters of the reads were removed with S-MART (Zytnicki and Quesneville, 2011) tools, then sequences were mapped using Mosaik (<http://code.google.com/p/mosaik-aligner/>) and Bowtie (Langmead et al., 2009) to the TAIR10 assembly. Only unique reads with at most 2 mismatches to the genome were kept. Graphs were produced with S-MART tools and *ad hoc* scripts. Data were normalized with respect to Col-0 under the assumption that the 16 canonical miRNA genes (miR156, 157, 159, 160, 162, 164, 165, 166, 167, 168, 169, 170, 171, 172, 319, 390) were globally unaffected (Figure 2). The normalization of *dcl2/dcl3/dcl4*, which showed the most drastic small RNA down-regulation, was further confirmed by RNA gel blot. MicroRNA annotation was downloaded from

RFAM (Burge et al., 2013). EndoIR-siRNAs were annotated using the sRNA-producing loci predicted by (Lindow et al., 2007), which did not match RFAM miRNAs. Loci producing siRNAs in a PolIV-dependent manner were retrieved from (Mosher et al., 2008). Young miRNAs were defined as mutant *dcl1*-independent sRNA: we downloaded the sRNAs produced by (Lu et al., 2006) and discarded all of those miRNAs that contained at least one read from this data set. The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no.XXX).

Plant protein sequences homologous to Arabidopsis RTL1 and RTL2 were retrieved using TblastN. DCL sequences were discarded based on the presence of RNA helicase and PAZ domains upstream of the RNaseIII and DRB domains. In each species, the RTL1 ortholog was identified based on the presence of a single DRB domain and a higher similarity to Arabidopsis RTL1 than to RTL2 in the RNaseIII domain. Proteins were aligned using MultiAlign.

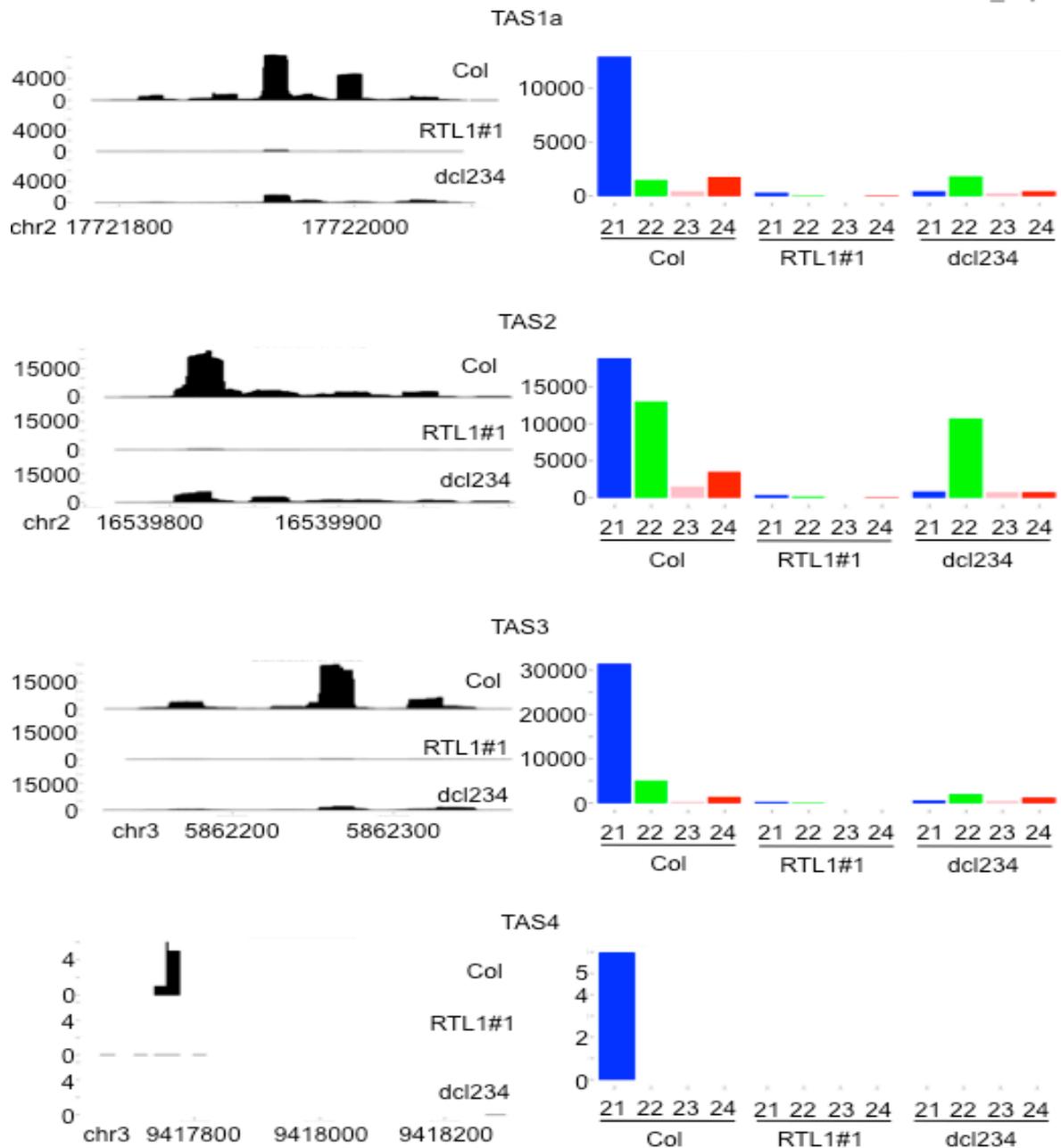
### **Acknowledgements**

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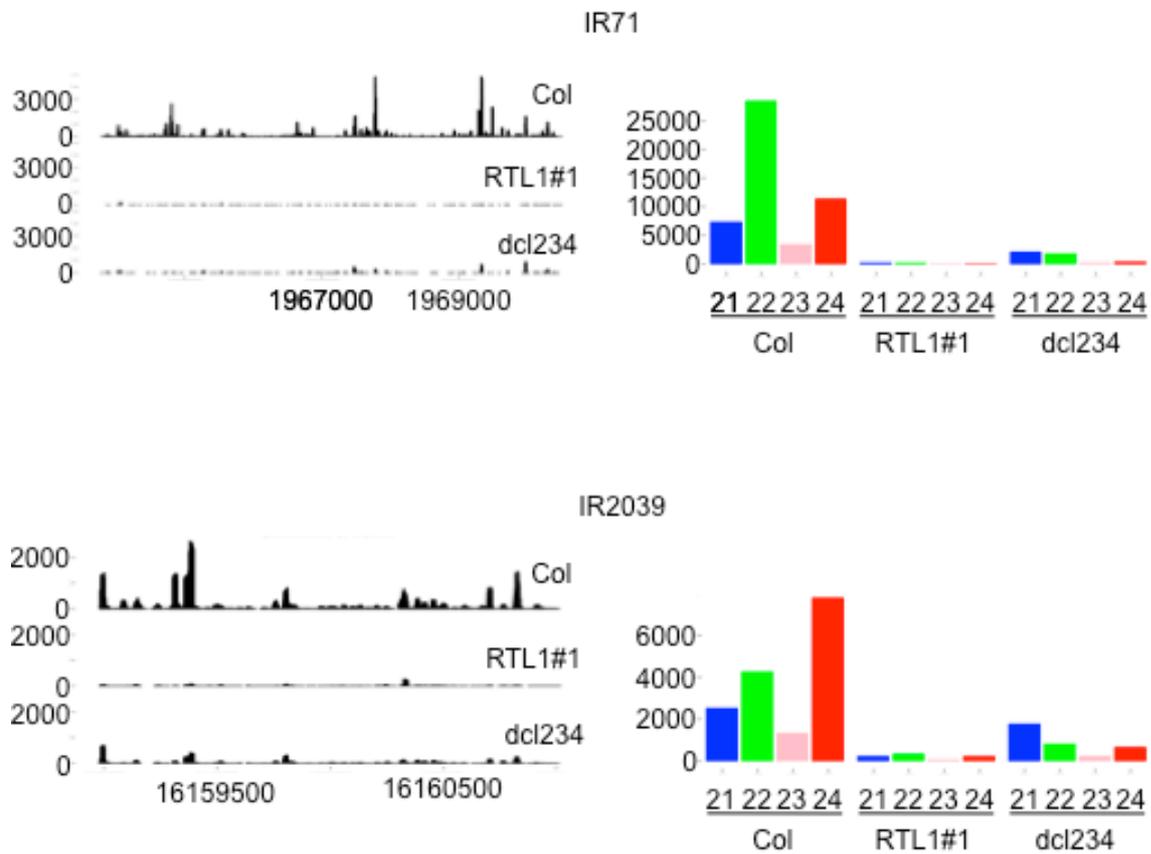
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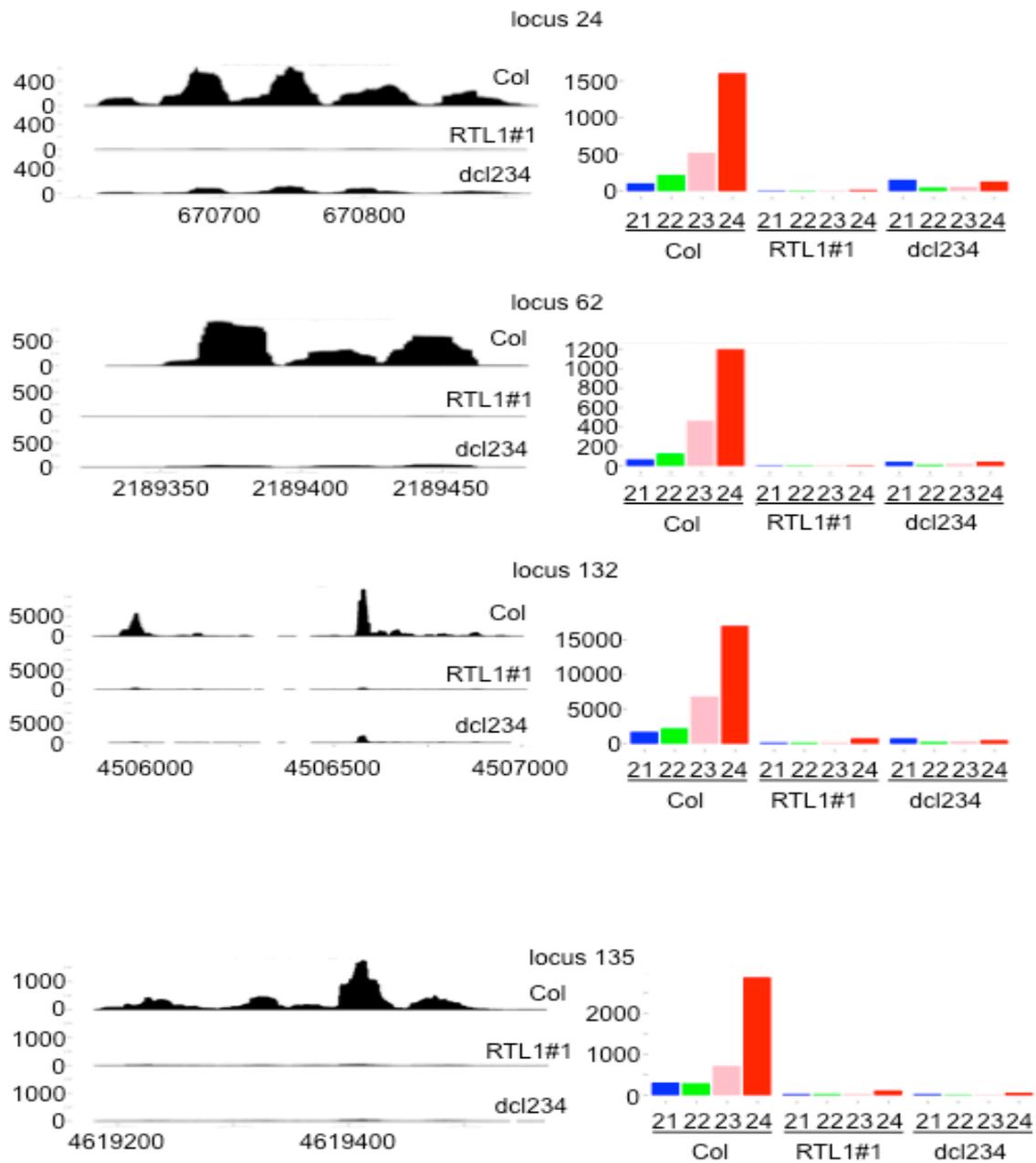
**Figure S1: Normalized abundance and distribution of four *TASiRNA* loci.**

Small RNA abundance was normalized to the total amount of conserved miRNAs. The distribution of *tas*iRNAs along the chromosome is shown on the left, and the size distribution is shown on the right. The sizes are indicated by different colors: 21-nt (blue), 22-nt (green), 23-nt (pink) and 24-nt (red).



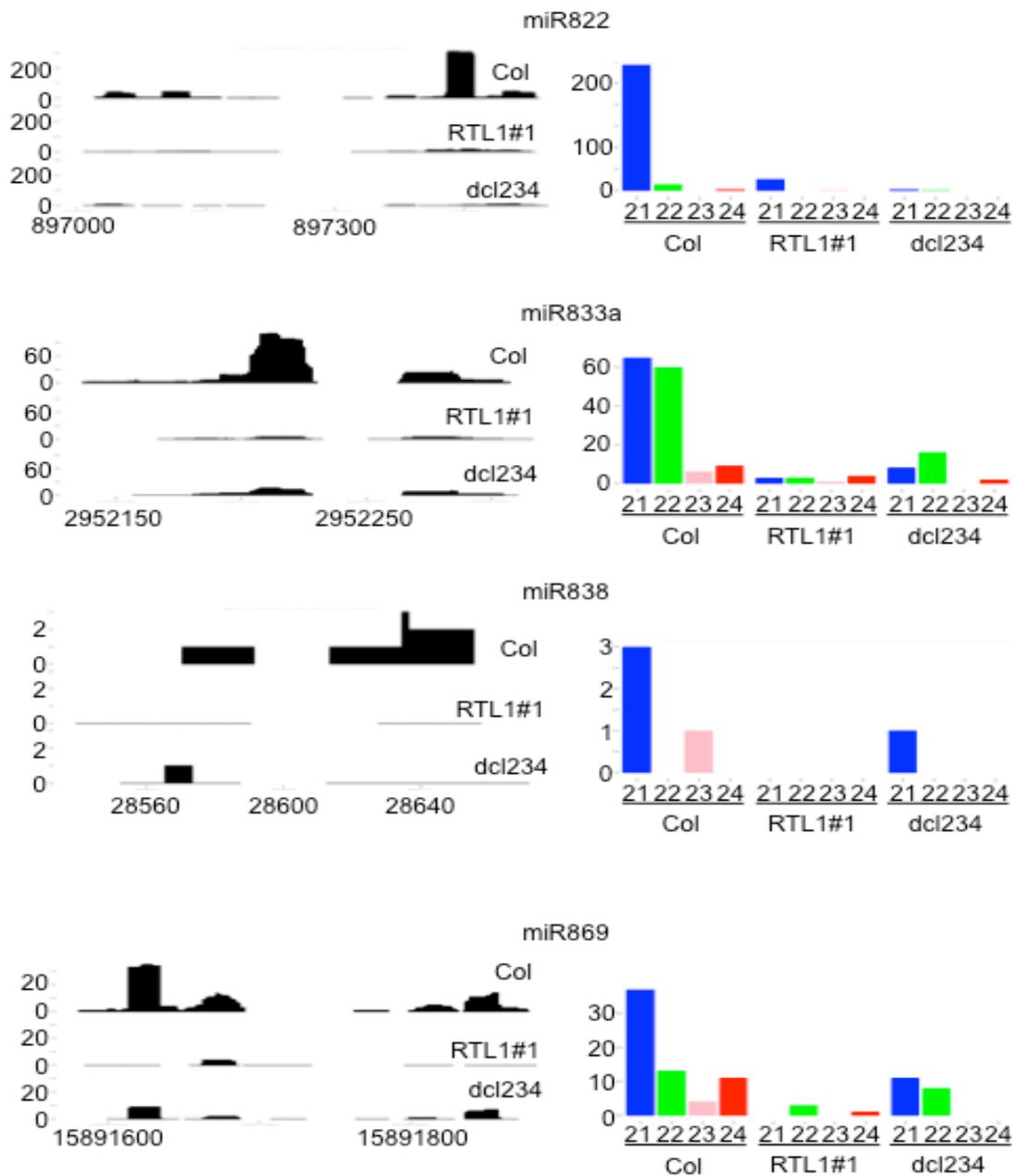
**Figure S2: Normalized abundance and distribution of two *EndoIR-siRNA* loci.**

Small RNA abundance was normalized to the total amount of conserved miRNAs. The distribution of *endoIR-siRNAs* along the chromosome is shown on the left, and the size distribution is shown on the right. The sizes are indicated by different colors: 21-nt (blue), 22-nt (green), 23-nt (pink) and 24-nt (red).



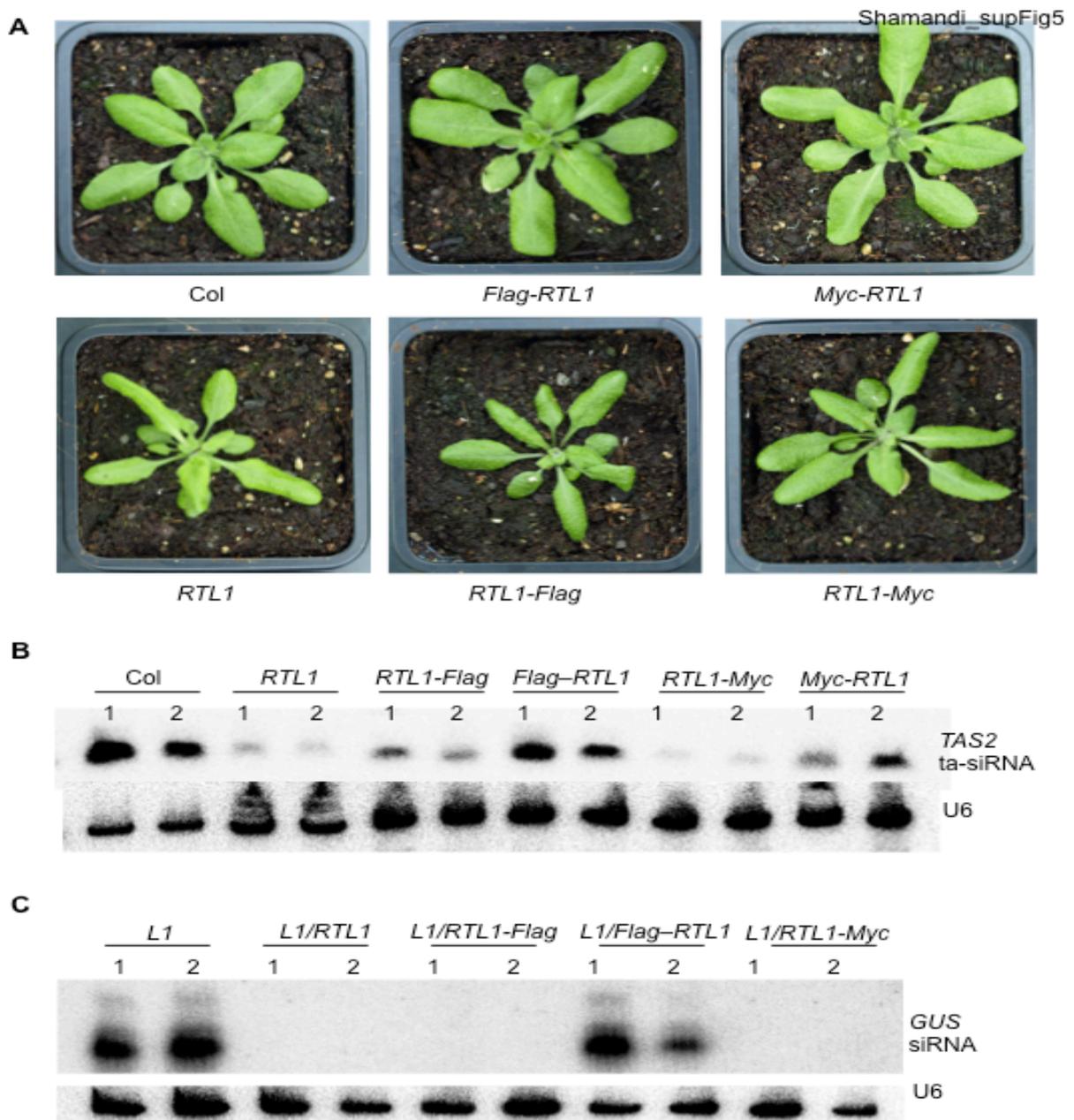
**Figure S3: Normalized abundance and distribution of four *PolIV/PolV*-siRNA loci.**

Small RNA abundance was normalized to the total amount of conserved miRNAs. The distribution of *PolIV/PolV*-siRNAs along the chromosome is shown on the left, and the size distribution is shown on the right. The sizes are indicated by different colors: 21-nt (blue), 22-nt (green), 23-nt (pink) and 24-nt (red).



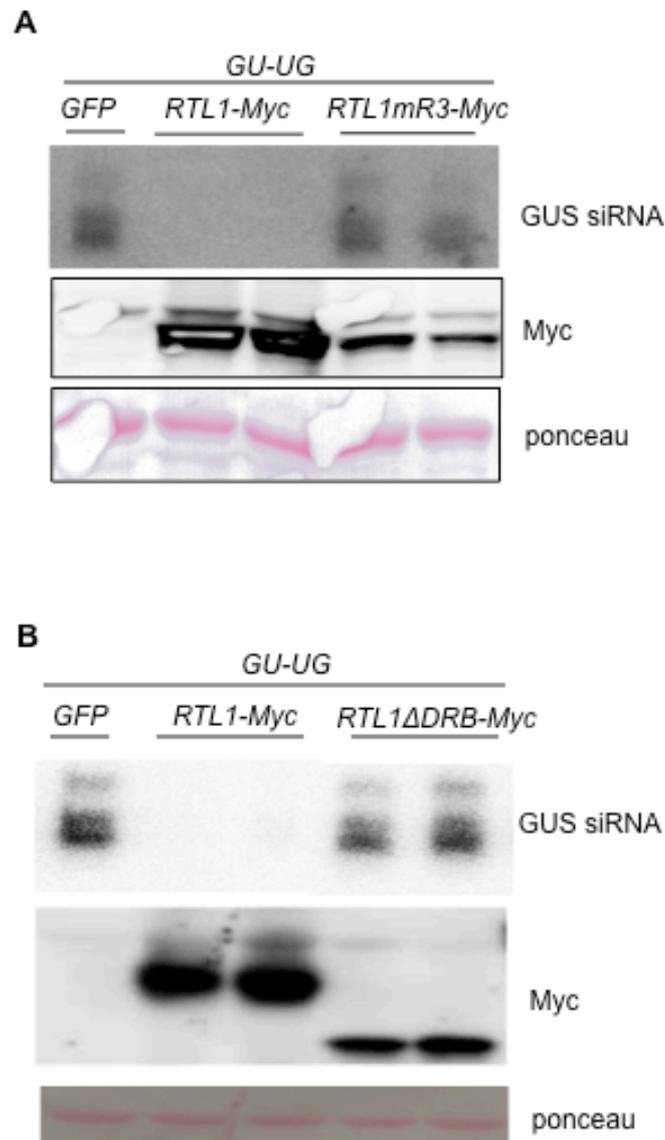
**Figure S4: Normalized abundance and distribution of four young *MIRNA* loci.**

Small RNA abundance was normalized to the total amount of conserved miRNAs. The distribution of young miRNAs along the chromosome is shown on the left, and the size distribution is shown on the right. The sizes are indicated by different colors: 21-nt (blue), 22-nt (green), 23-nt (pink) and 24-nt (red).



**Figure S5: Effect of tagged-*RTL1* expression on development and PTGS.**

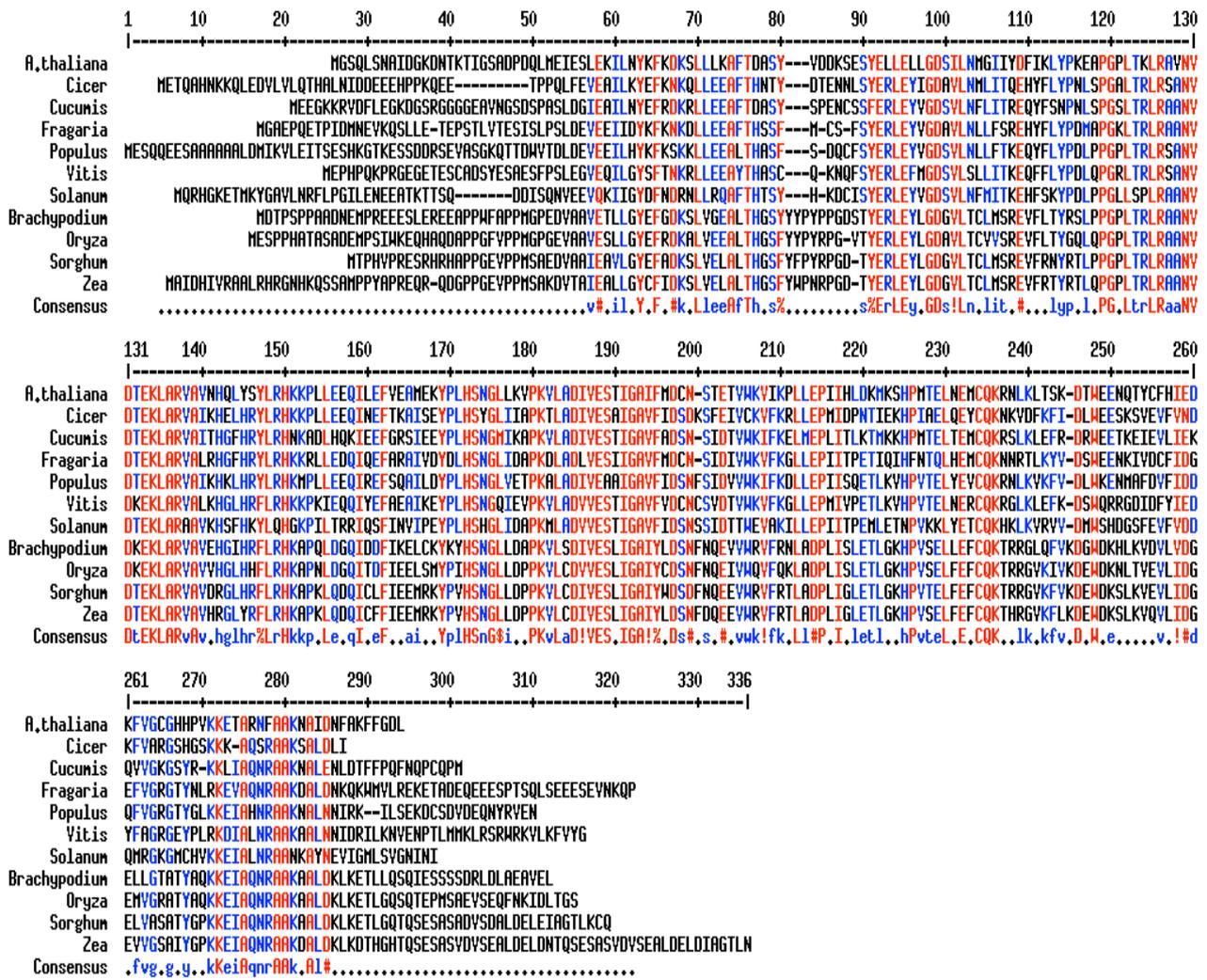
A) Representative phenotypes of wild-type plants (Col) and transgenic plants expressing the indicated tagged-*RTL1* constructs. B) Total RNA was extracted from two independent transformants expressing each of the indicated tagged-*RTL1* constructs and hybridized with a *TAS2* probe and with *U6* as a loading control. C) The same constructs were introduced into line *L1*. Total RNA was extracted from two independent transformants expressing each of the indicated tagged-*RTL1* constructs and hybridized with a *GUS* probe and with *U6* as a loading control.



**Figure S6: Mutagenesis of the RTL1 RNaseIII and DRB domains impairs RTL1 activity.**

*Nicotiana benthamiana* leaves were infiltrated with a 35S::GU-UG construct (GU-UG) together with either a wild-type tagged 35S::RTL1-Myc construct (RTL1-Myc), a construct mutated in the RNaseIII domain (RTL1mR3-Myc) (A), or a construct with a deleted DRB domain (RTL1ΔDRB-Myc) (B), or a 35S::GFP control (GFP). Low molecular weight (LMW) RNAs were hybridized with a GUS probe and with U6 as a loading control. Proteins were extracted and hybridized with a Myc antibody. Ponceau staining serves as a loading control.





**Figure S8: Alignment of plant RTL1 amino acid sequences.**

Alignment of RTL1 amino acid sequences from *Arabidopsis thaliana* ([NP\\_680697.4](#)), *Brachypodium distachyon* ([XP\\_003560753.1](#)), *Cicer arietinum* ([XP\\_004502137.1](#)), *Cucumis sativus* ([XP\\_004159229.1](#)), *Fragaria vesca* ([XP\\_004287969.1](#)), *Oryza sativa* ([NP\\_001057601.1](#)), *Populus trichocarpa* ([XP\\_002301611.1](#)), *Solanum lycopersicum* ([XP\\_004243532.1](#)), *Sorghum bicolor* ([XP\\_002444209.1](#)), *Vitis vinifera* ([XP\\_002270948.2](#)), *Zea mays* ([ACG34313.1](#)).

Supplemental Table 1. Oligonucleotide sequences

Name	DNA Sequence
Oligonucleotides used for RT and PCR amplification	
attB1RTL1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGGATCGCAACTCTCAAA
attB2RTL1R	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAGGTCTCCGAAAAATTTAGC
attB1RTL2F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGGATCACTCTATCTCACC
attB2RTL2R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGATAATGAGATTTTCTCAAGG
attB2RTL1-R3-R	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTCTAAATGTATTATTGGCTCCAAC
RTL1-6	GTTCTGTCATTGGATGGCTC
RTL1-7	CCAAAACCATCGGATCAGCGG
RTL1m2-F	GAATCGTACGCACTCCTAGAACTTTTAGGAGCTTCGATCC
RTL1m2-R	GGATCGAAGCTCCTAAAAGTTCTAGGAGTGCCTACGATTC
5rtl1NdeI	CATATGAACAAGACACAAACAC
3rtl1XhoI	CTCGAGAAGGTCTCCGAAAAATTTAGC
IR71fwd1	AAATGACCGCTACACTGCTTATCT
IR71rev1	TCTCTCGTCAATGGACAATGAATC
IR71fwd2	TAGGAAAGCTCGAACCCCTTGG
IR71rev2	TGAATGGTAATGATTTGTCTC
IR71rt	GATATGAGGTGCTTTGAATGG
U3fwd	ACGGACCTTACTTGAACAGGATCTG
U3rt	CTGTCAGACCGCCGTGCGA
Oligonucleotides used as probes for small RNA detection	
miR159	TAGAGCTCCCTTCAATCCAAA
miR160	TGGCATAACAGGGAGCCAGGCA
miR173	GTGATTTCTCTCTGCAAGCGAA
miR167	TAGATCATGCTGGCAGCTTCA
miR164	TGCACGTGCCCTGCTTCTCCA
miR156	GTGCTCACTCTCTTCTGTCA
miR839	GGGAACGATGAAAGGTTGGTA
amiR-RTL1a	AAGAGTGAATCGTACGAACTA
amiR-RTL1b	CGGGTCCCTGTAATCACCAA
TAS1	TACGCTATGTTGGACTTAGAA
TAS2	GTGCTTCACAATGCTCTTTC
siR1003	ATGCCAAGTTTGGCCTCACGGTCT
siR02	GTTGACCAGTCCGCCAGCCGAT
IR71	TCCTTTCCCTTTCCCTTTCTAC
U6	TTGCGTGCATCCTTGCGCAGG

## 3.1.2 - Supplementary Results

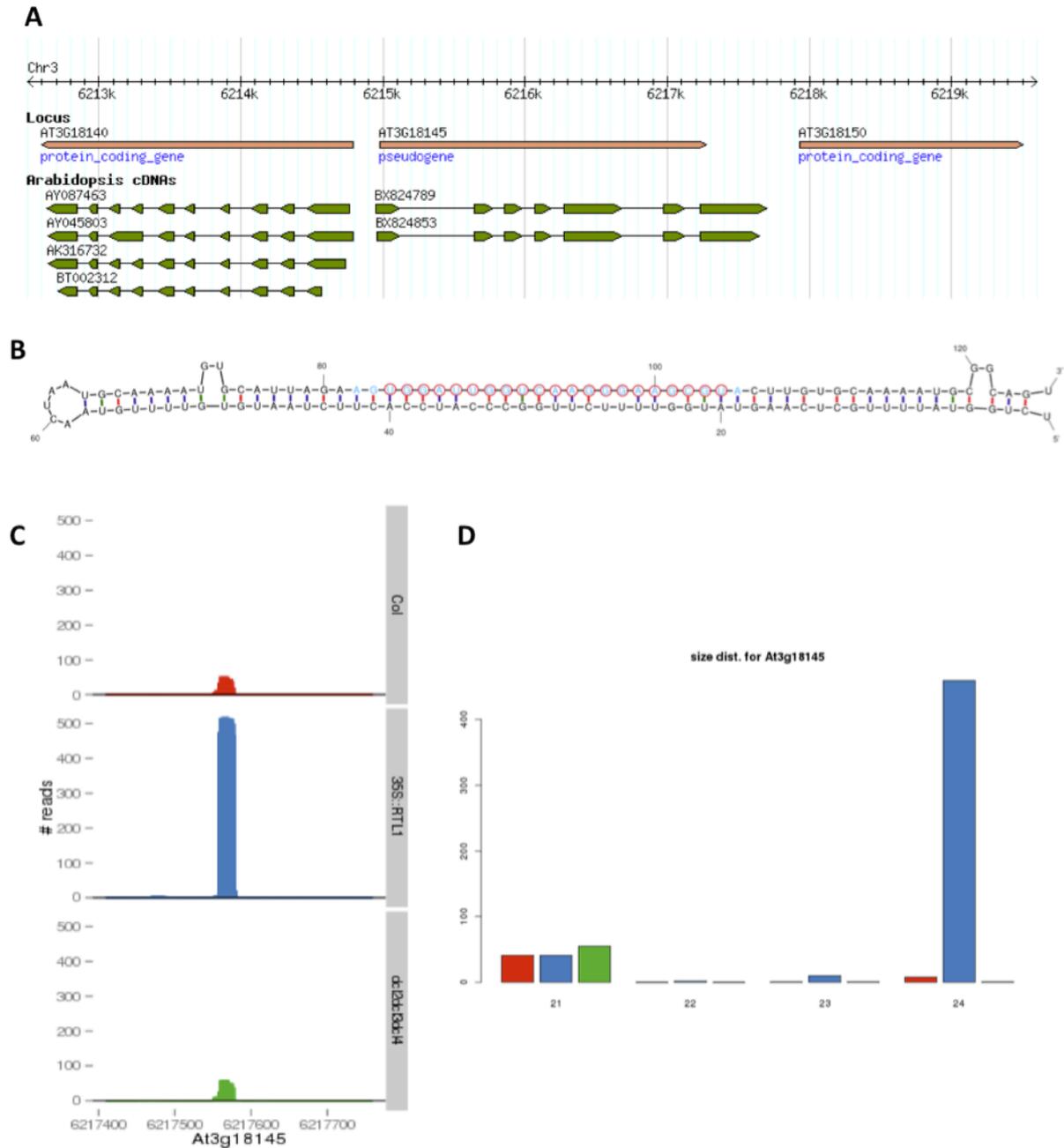
### 3.1.2.1 - RTL1 exceptionally helps producing siRNAs

Although, siRNAs are generally and drastically more infrequent in *35S::RTL1* plants, siRNAs are sometimes differently distributed along small RNA-generating loci or are found in different sizes compared to wild-type controls. This suggests that long dsRNA precursor molecules are not always degraded in *35S::RTL1* plants. Rather, long dsRNA precursor molecules could sometimes be processed by a different DCL after an initial processing by RTL1. Such an example is found at the *At3g18145* locus (Figure 3.1.A). In wild-type plants, the 3'UTR of the *At3g18145* mRNA is predicted to fold into a long hairpin (Figure 3.1.B), and gives rise to very low amounts of 21-nt miR3440b undetectable by RNA gel blot (Figures 3.1.C, 3.1.D and 3.2.A).

The fact that this miRNA accumulates at similar level in wild-type plants and in the *dcl2 dcl3 dcl4* triple mutant (Figures 3.1.C and 3.1.D) indicates that this hairpin is processed by DCL1. However, the low abundance of miR3440b and the fact that DCL1 normally processes imperfectly paired miRNA precursors suggests that the long near perfectly paired 3'UTR of the *At3g18145* mRNA is likely not an efficient substrate of DCL1.

In contrast to wild-type plants, *35S::RTL1* plants accumulate a high amount of a unique 24-nt *At3g18145* siRNA molecule (Figures 3.1.C and 3.1.D), which is detectable by RNA gel blot (Figure 3.1.E). This result suggests that RTL1 processes the 3'UTR of the *At3g18145* mRNA, thus generating a molecule that can be a substrate for another DCL, which is likely DCL3 given that the siRNA is 24-nt in length. This hypothesis is supported by the fact that this 24-nt siRNA accumulates in *35S::RTL1* plants, but not in the *dcl2 dcl3 dcl4* triple mutant transformed with the *35S::RTL1* construct (Figure 3.2.A).

Moreover, *N. benthamiana* leaves infiltrated with *35S::At3g18145* + *35S::RTL1-Myc* accumulated a large amount of this 24-nt siRNA (Figure 3.2.A). Consistent with its dependence on RTL1 overexpression, this 24-nt siRNA is not detected in *35S::GU-UG* + *35S::GFP* infiltrated *N. benthamiana* leaves, and only low amounts of 21- and 24-nt siRNAs are detected in *N. benthamiana* leaves infiltrated with *35S::At3g18145* + *35S::GFP* (Figure 3.2.B). This can be explained by the action of DCL4 and DCL3, which can process every transiently expressed 35S-driven transgene, into 21- and 24-nt siRNA respectively. *N. benthamiana* leaves infiltrated with *35S::At3g18145* + *35S::RTL1mR3* accumulated less 21-nt siRNA and 24-nt siRNAs than leaves infiltrated with *35S::At3g18145* + *35S::GFP*, suggesting that the RTL1mR3 protein, which lacks RNaseIII activity, can compete with DCL4 and DCL3, most probably by the binding of its DRB domain to *At3g18145* dsRNA, thus limiting the production of siRNA by these DCLs.



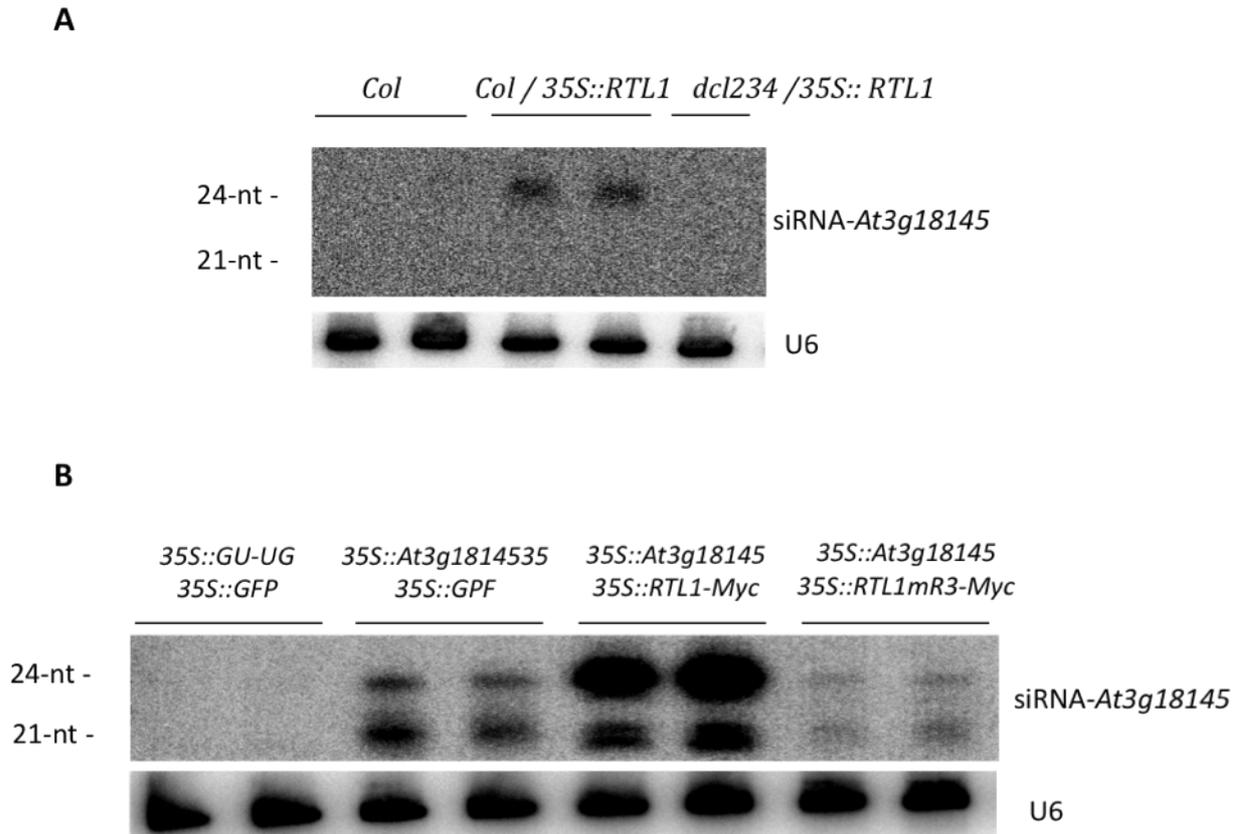
**Fig. 3.1** *35S::RTL1* plants accumulate a 24-nt siRNA from the 3'UTR of *At3g18145*

**A)** TAIR annotation of the *At3g18145* locus

**B)** Predicted hairpin structure of the *At3g18145* 3' UTR. Nucleotides of the sequence corresponding to the 24-nt siRNA are indicated in blue. Nucleotides of the sequence corresponding to the 21-nt miRNA 3440b are circled in red.

**C)** Chromosomal distribution of small RNAs in *Col*, *35S::RTL1* and *dcl2 dcl3 dcl4*.

**D)** Size distribution of small RNAs in *Col*, *35S::RTL1* and *dcl2 dcl3 dcl4*.



**Fig 3.2: RTL1 promotes the accumulation of 24-nt siRNA from At3g18145.**

**A)** Total RNA was extracted from flowers of wild-type *Col*, transgenic *Col* carrying the *35S::RTL1* construct, or transgenic triple mutant *dcl2 dcl3 dcl4 (dcl234)* carrying the *35S::RTL1* construct. RNA gel blots were hybridized with an oligo probe corresponding to the *At3g18145* 3'UTR-derived 24-nt siRNA identified in Arabidopsis *35S::RTL1* plants. U6 serves as a loading control.

**B)** *N.benthamiana* leaves were infiltrated with the *35S::At3g1814535* construct + *35S::RTL1-Myc* or *35S::RTL1mR3-Myc* or *35S::GFP* as a control. RNA gel blots were hybridized with an oligo probe corresponding to the *At3g18145* 3'UTR-derived 24-nt siRNA identified in Arabidopsis *35S::RTL1* plants. U6 serves as a loading control.

### **3.1.2.2– RTL1 over expression doesn't seem to affect the susceptibility of plants to bacterial infections**

Different types of small RNAs have been reported to be up or down regulated during bacterial infections and to play roles in plant immunity responses (Fahlgren et al., 2007; Katiyar-Agarwal et al., 2006; Navarro et al., 2008). Considering the impact of RTL1 overexpression on the siRNA population, we wondered if RTL1 could affect plant susceptibility during bacterial infections. To test this idea, leaves of Arabidopsis Col and *35S::RTL1-Flag* were infiltrated with *Pseudomonas syringe* DC3000 AvrRpm1 or *Edwina amylovora* CFBP1430.

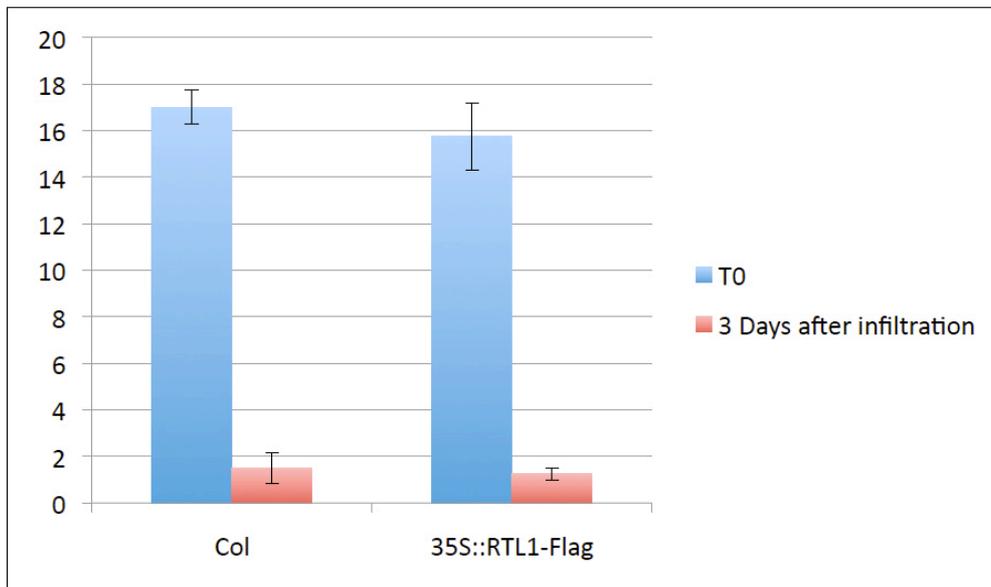
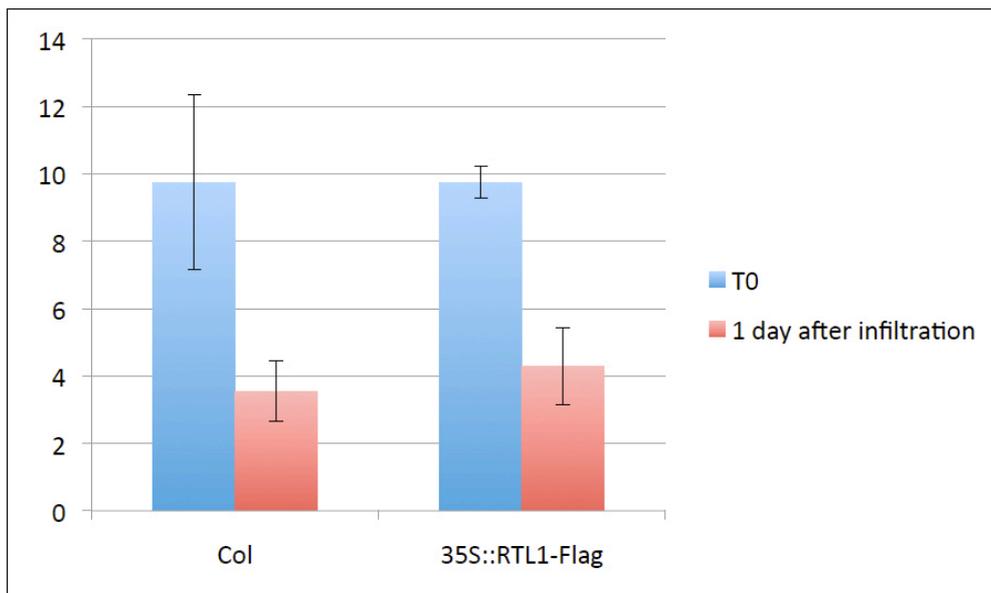
Grinded infected leaves were used for serial dilutions, followed by plating on an LB medium and counting of the colonies. For plants, which were inoculated with *P.syringe* DC3000 AvrRpm1, in both Col and *35S::RTL1-Flag*, the number of colonies at T0 (just after infiltration) was similar (Fig 3.3.A), suggesting that the quality of infiltration in both was identical. Moreover the number of colonies 3 days after infiltration for both Col and *35S::RTL1*, was lower than T0; showing that the plant's initial immune system was activated due to bacterial infection. Finally, there was no significant differences between the number of the colonies at 3 days after infiltrations between Col and *35S::RTL1-Flag* which means the overexpression of RTL1 in Arabidopsis plants has no significant impact on plants susceptibilities to *P. syringe* (Fig.3.3.A).

For plants infiltrated with *E. solanacearum*, the same results were obtained. No significant change between Col and *35S::RTL1-Flag*, was observed (Fig. 3.3.B). Altogether it seems that RTL1 doesn't interfere with plant responses to bacterial infections, at least for the two bacteria tested.

### **3.1.2.3 – Attempts to down-regulate RTL1 using artificial miRNAs**

Because *rtl1* null alleles could not be found in publicly available libraries, we decided to use artificial miRNAs to specifically target *RTL1* mRNA. Two different constructs were generated to produce amiR-RTL1a and amiR-RTL1b using the constitutive pUBQ10 promoter. At first, the accumulation of both amiR-RTL1a and amiR-RTL1b was confirmed by northern blot analysis in *N.benthamiana* agroinfiltrated leaves (Fig. 3.4.A & 3.4.B).

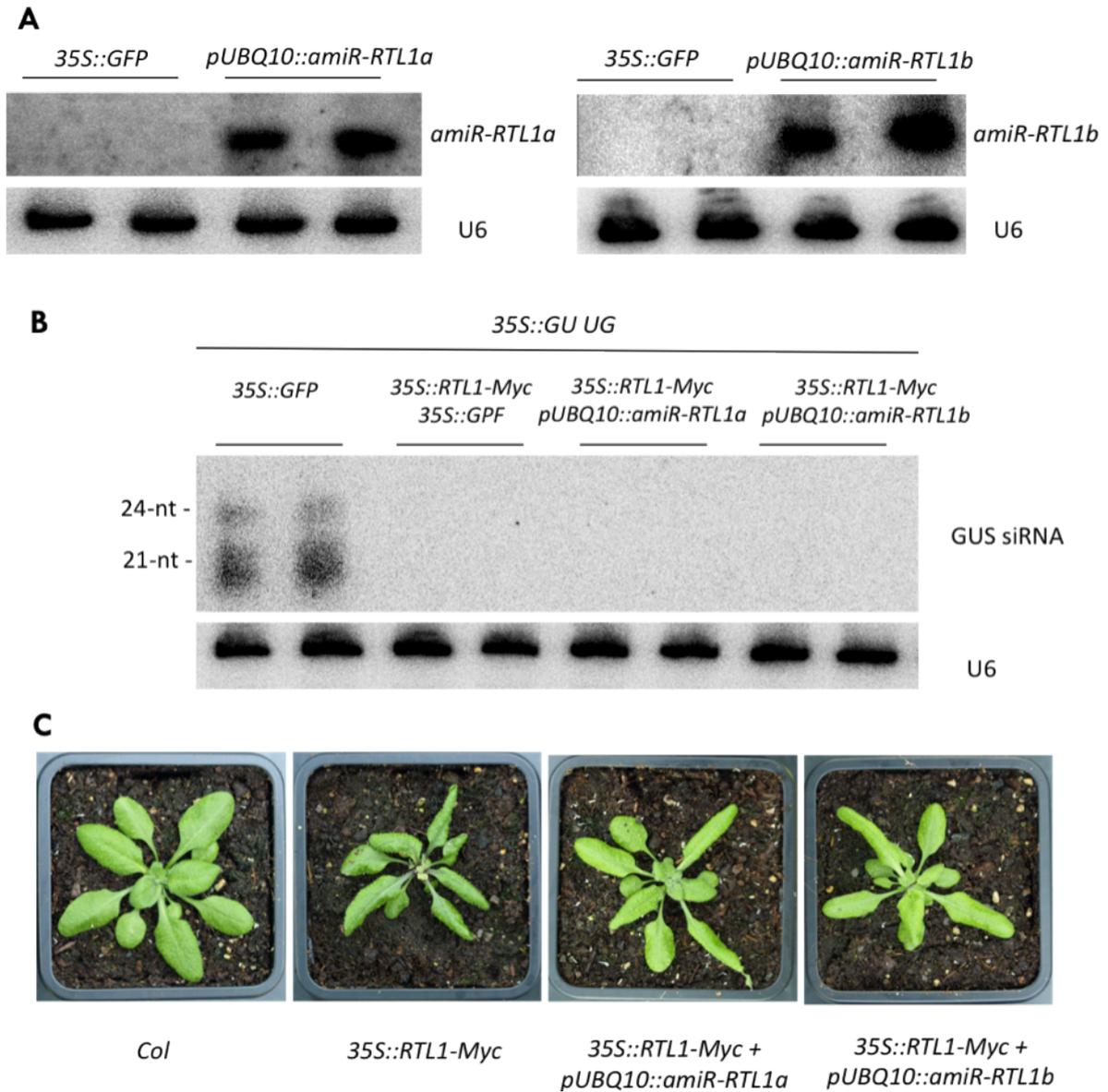
Because RTL1 is only very weakly expressed in roots, we anticipated that it would be difficult to monitor a decrease in *RTL1* mRNA accumulation in roots of transgenic Arabidopsis expressing these amiRs. Thus, we first tested if these amiRs were capable of suppressing the accumulation of *RTL1* mRNA in a transient assay. For this purpose, we co-infiltrated *35S::GU-UG* + *35S::RTL1* + *pUBQ10::amiR-RTL1a* or *pUBQ10::amiR-RTL1b*. The results showed that, even though we were able to detect the accumulation of amiR-RTL1a and amiR-RTL1b, neither of them was able to inhibit the action of RTL1 on the production of GU siRNAs (Fig. 3.4.C). In a second experiment, we transformed

**A****B**

**Fig. 3.3 Effect of RTL1 overexpression on bacterial infection.**

**A)** Leaves of wild-type *Arabidopsis* control (Col) and transgenic *35S::RTL1-Flag* plants were infiltrated with *Pseudomonas syringae* DC3000 AvrRpm1. The average number of bacterial colonies growing on LB mediums from 30  $\mu$ l of  $10^{-3}$  dilutions of grinded individual leaves suspended in water was determined at T0 (just after infiltration) or three days after infiltration.

**B)** Leaves of wild-type *Arabidopsis* control (Col) and transgenic *35S::RTL1-Flag* plants were infiltrated with *Erwinia amylovora* CFBP1430. The average number of bacterial colonies growing on LB mediums from 30  $\mu$ l of  $10^{-4}$  dilutions of grinded individual leaves suspended in water was determined at T0 (just after infiltration) or one day after infiltration.



**Fig. 3.4: Effect of artificial miRNA directed against RTL1.**

**A)** *N.benthamiana* leaves were infiltrated with the *pUBQ10::amiR-RTL1a* or *pUBQ10::amiR-RTL1b* construct. RNA gel blots were hybridized with oligo probes complementary to *amiR-RTL1a* or *amiR-RTL1b*. U6 serves as a loading control.

**B)** *N.benthamiana* leaves were infiltrated with the *35S::GU-UG* construct + *pUBQ10::amiR-RTL1a* or *pUBQ10::amiR-RTL1b* construct or *35S::GFP* as a control. RNA gel blots were hybridized with a GU probe. U6 serves as a loading control.

**C)** Phenotypes of Arabidopsis transformants carrying the *35S::RTL1-Myc* construct alone or *35S::RTL1-Myc + pUBQ10::amiR-RTL1a* or *pUBQ10::amiR-RTL1b*.

Arabidopsis plants with *35S::RTL1-Myc* + *pUBQ10::amiR-RTL1a* or *pUBQ10::amiR-RTL1b*. All transformants exhibited the typical zippy phenotype of *35S::RTL1-Myc* plants (Fig. 3.4.D), suggesting that amiR-RTL1a and amiR-RTL1b are unable to target *RTL1* mRNA, at least when produced from the 35S promoter. Indeed, it is possible that the amiR-RTL1a and amiR-RTL1b are unable to suppress *RTL1* simply because the 35S promoter driving *RTL1* is stronger than the pUBQ10 promoter driving the amiR-RTL1 constructs.

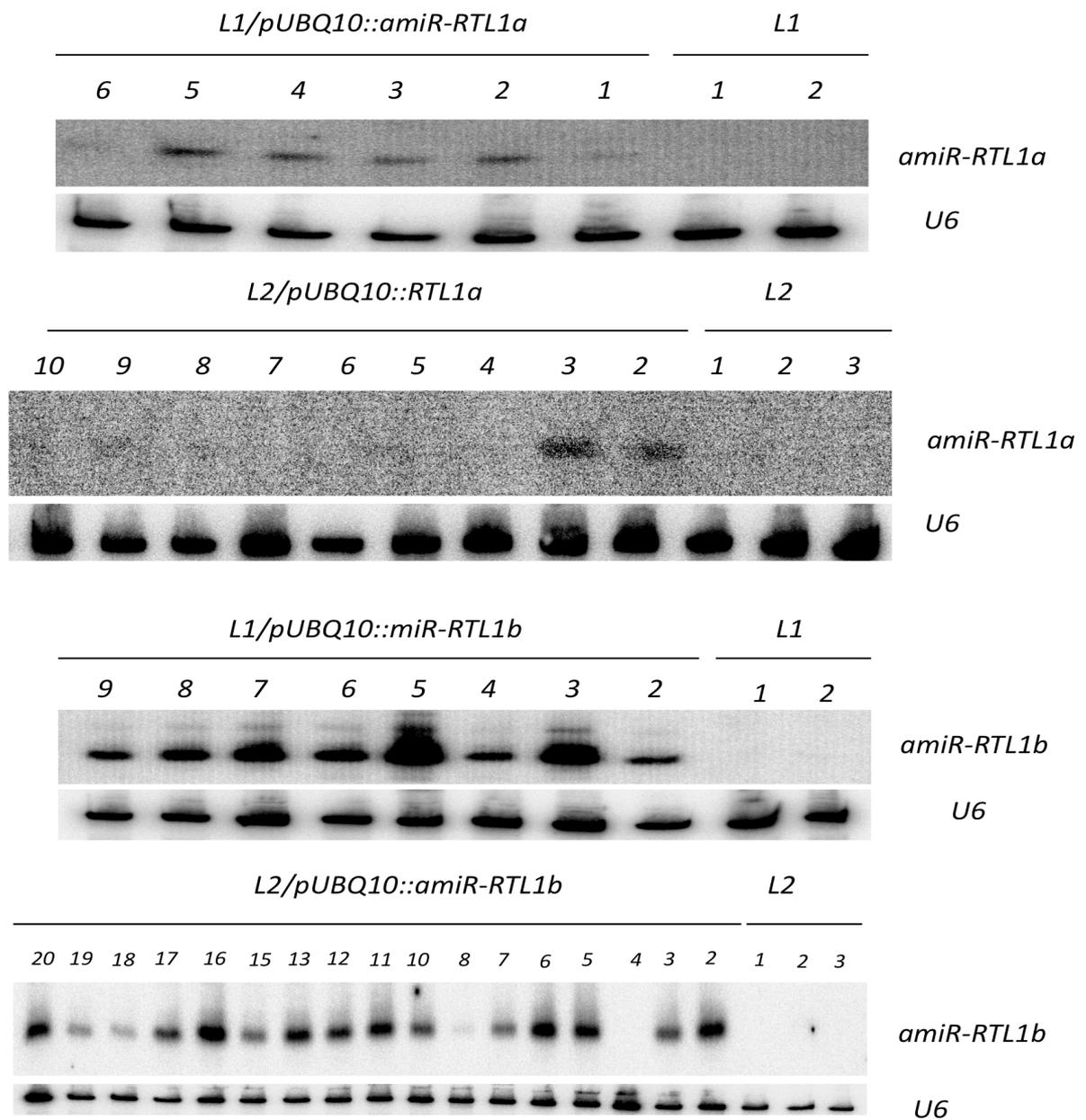
To determine if amiR-RTL1a or amiR-RTL1b could target the endogenous *RTL1* mRNA when expressed at physiological level, we transformed the *L1* and *L2* lines with the *pUBQ10::amiR-RTL1a* and *pUBQ10::amiR-RTL1b* constructs. The *L1* and *L2* lines each carry a *35S::GUS* reporter transgene that undergoes PTGS with 100% efficiency (Elmayan et al., 1998). However, PTGS completely suppresses *GUS* mRNA accumulation in leaves of these lines, but not in roots (Elmayan and Vaucheret, unpublished data), suggesting that the expression of RTL1 in roots could contribute to partially impairing PTGS in this tissue. Therefore, we anticipated that the expression of amiR-RTL1a or amiR-RTL1b under the pUBQ10 promoter could down-regulate *RTL1* mRNA accumulation and thus enhance PTGS efficiency in roots to the level observed in leaves. *L1* and *L2* transformants accumulating the highest levels of amiR-RTL1a or amiR-RTL1b (Figure 3.5) were grown in hydroponic conditions, and GUS enzymatic activity was measured in roots (Figure 3.6.A & 3.6.B). One *L1/pUBQ10::amiR-RTL1b*, one *L2/pUBQ10::amiR-RTL1a* and one *L2/pUBQ10::amiR-RTL1b* plants exhibited a level of GUS enzymatic activity significantly lower than that observed in *L1* and *L2* controls, suggesting that these lines could have reduced RTL1 activity.

Homozygous siblings will be identified to confirm this result. If it is confirmed, these plants will be used to determine if RTL1 down-regulation affects virus-sensitivity in the opposite way to RTL1 over-expression.

## 3.2 – RTL2

RTL2 contains one RNaseIII domain and two DRB domains and is ubiquitously expressed. RTL2 is able to cleave the 3' External Transcribed Spacer (EST) of ribosomal 45S-pre-rRNA *in vitro* and *in vivo* (Comella et al., 2008). Another *in vitro* assay indicates that AtRTL2 recognizes and cleaves dsRNA specifically and can cleave higher-order structures of ssRNAs and also causes the high accumulation of 21 nt- siRNAs *in-vivo* (Kiyota et al., 2011).

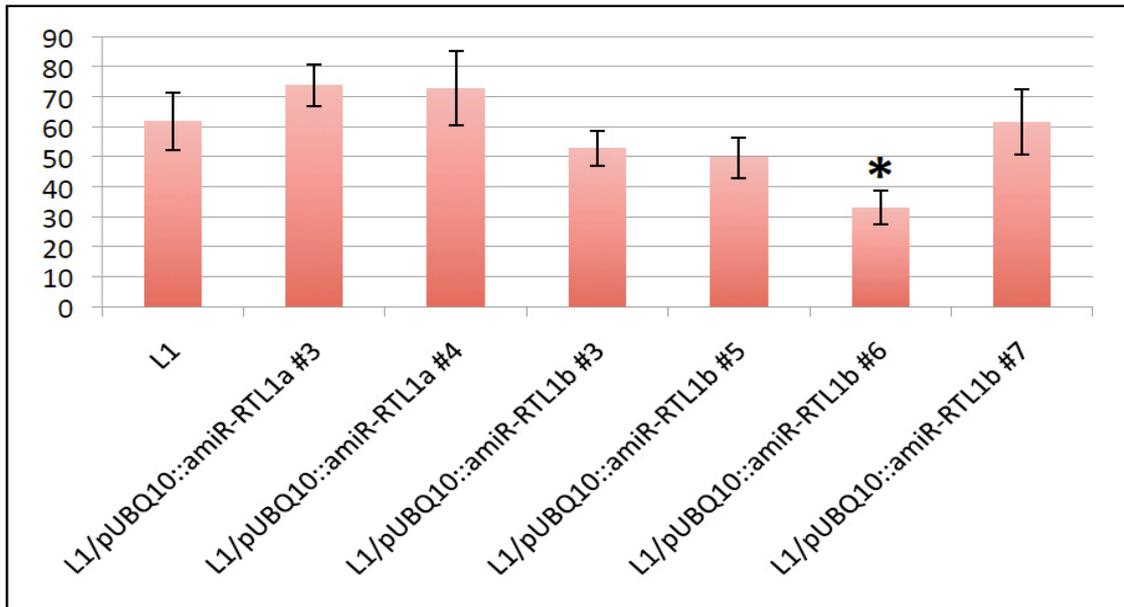
Neither *rtl2* mutants nor *35S::RTL2* plants exhibit consistent developmental defects under standard greenhouse growth conditions, and their small RNA profile is comparable to that of the wild-type control. Indeed, the 23-24-nt reads represented 90% of the small RNA reads, and the distribution of unique siRNA reads along the five chromosomes is similar to that of wild-type plants, suggesting that



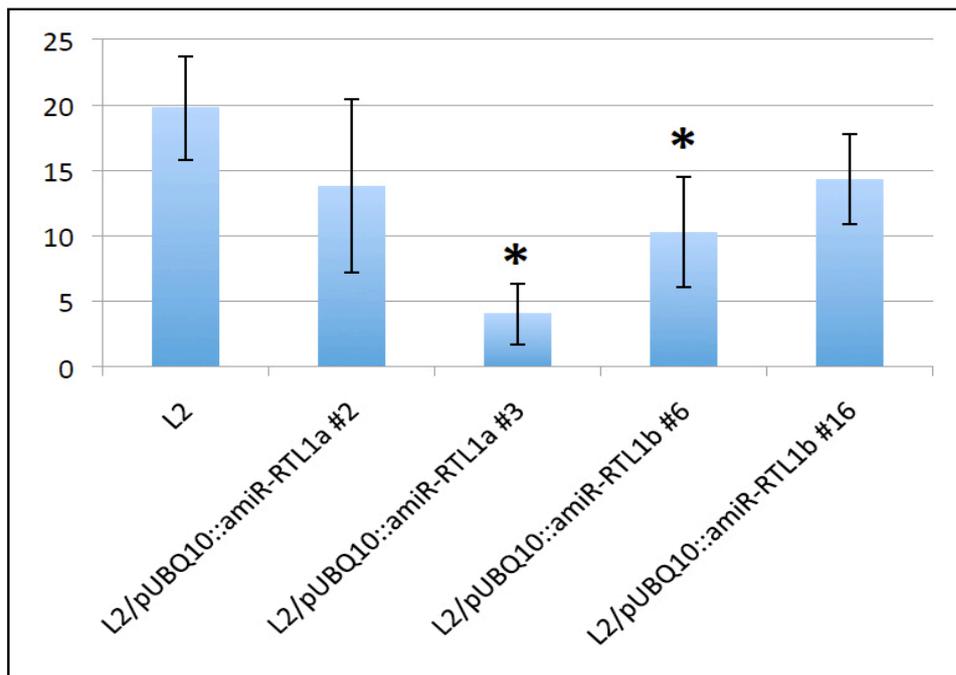
**Fig. 3.5 Detection of amiR-RTL1a and amiR-RTL1b in L1 and L2 transformants.**

Total RNA was extracted from flowers of individual *L1* and *L2* transformants carrying the *pUBQ10::amiR-RTL1a* or *pUBQ10::amiR-RTL1b* construct. RNA gel blots were hybridized with oligo probes complementary to amiR-RTL1a or amiR-RTL1b. U6 serves as a loading control.

**A**



**B**



**Fig. 3.6 GUS activity in roots of plants expressing amiR-RTL1a or amiR-RTL1b**

**A)** *L1* control plants and transformants.

**B)** *L2* control plants and transformants.

The \* indicates plants with a significantly lower level of GUS activity than the *L1* or *L2* control.

RTL2 has no major contribution to the small RNA repertoire (see article). Nevertheless, we further looked at RTL2's action using in vivo approaches. The results obtained are detailed below.

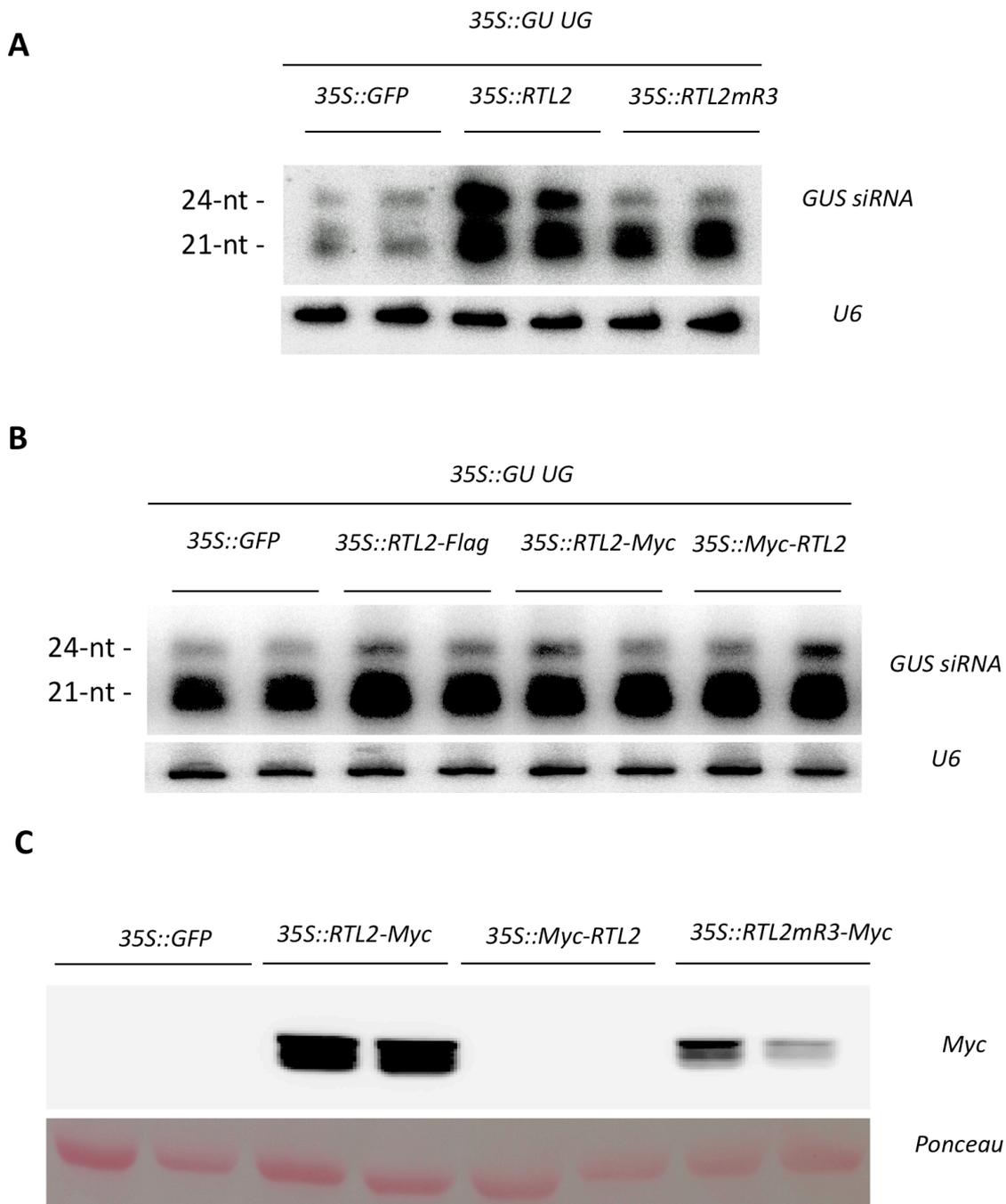
### **3.2.1 - RTL2 enhances the accumulation of 21- and 24-nt siRNAs**

The effect of RTL2 overexpression on transgene-derived siRNAs was tested using *N. benthamiana* Agroinfiltration. The 35S::*GU-UG* transgene expressing an inverted repeat hairpin RNA consisting of the 5' part of the GUS reporter sequence (GU) was introduced transiently into *N. benthamiana* leaves together with either a 35S::*RTL2* or a 35S::*GFP* control. 35S::*GU-UG* + 35S::*RTL2* infiltrated leaves accumulated higher levels of 21- and 24-nt GU siRNAs compared with 35S::*GU-UG* + 35S::*GFP* infiltrated leaves (Fig. 3.7.A), indicating that RTL2 enhances the accumulation of transgene siRNAs deriving from an inverted repeat.

This enhancing activity however, could only be observed when the RTL2 construct is without any additional tag proteins and has its original stop codon. Indeed, none of the combinations of RTL2, fused to Myc or Flag tag proteins at its N- or C-terminal affected the level of GU siRNA accumulation (Fig. 3.7.B). Moreover, western blot analysis revealed that an RTL2-Myc fusion protein is stable whereas the addition of a Myc tag at the N-terminus of RTL2 destabilizes the protein (Fig.3.7.C). Therefore, RTL2 enzymatic activity or protein stability can easily be perturbed by the presence of tags.

### **3.2.2 – Mutations in the RTL2 RNaseIII domain mostly affect the accumulation of 24-nt siRNAs**

To determine if an intact RNaseIII domain is required for RTL2 to enhance siRNA accumulation, the conserved catalytic site aminoacids E89 and D96 were both mutagenized to alanine (we refer to this transgene as RTL2mR3). Then, *N. benthamiana* leaves were co-infiltrated with 35S::*GU-UG* and either 35S::*RTL2* or 35S::*RTL2mR3*. 35S::*GU-UG* + 35S::*RTL2mR3* infiltrated leaves accumulated lower levels of 24-nt GU siRNAs but almost the same level of 21-nt GU siRNAs compared with 35S::*GU-UG* + 35S::*RTL2* infiltrated leaves. The level of 24-nt GU siRNA was similar to 35S::*GU-UG* + 35S::*GFP* control (Fig. 3.7.A), suggesting that the wild-type RNaseIII domain of RTL2 is required for RTL2 action on 24-nt siRNA but not on 21-nt. Although tagged RTL2 proteins appear inactive, western blot analyses were performed on *N. benthamiana* leaves infiltrated with 35S::*RTL2-Myc* and 35S::*RTL2mR3-Myc* to determine the effect of the RNaseIII mutations on protein stability. Results indicate that the RNaseIII mutations reduce protein stability (Fig.3.7.C), similar to what has been observed with RTL1. Whether the reduced amount of protein is responsible for the reduced amount of 24-nt remains to be determined.



**Fig.3.7 Effect of wild-type, mutant or tagged RTL2 on siRNA accumulation.**

**A)** *N.benthamiana* leaves were infiltrated with the *35S::GU-UG* construct + *35S::RTL2* or *35S::RTL2mR3* or *35S::GFP* as a control. RNA gel blots were hybridized with a GU probe. U6 serves as a loading control.

**B)** *N.benthamiana* leaves were infiltrated with the *35S::GU-UG* construct + the indicated tagged *35S::RTL2* constructs or *35S::GFP* as a control. RNA gel blots were hybridized with a GU probe. U6 serves as a loading control.

**C)** *N.benthamiana* leaves were infiltrated with the *35S::GU-UG* construct + the indicated tagged *35S::RTL2* constructs or *35S::GFP* as a control. Western blots were hybridized with a Myc probe. Ponceau staining serves as a loading control.

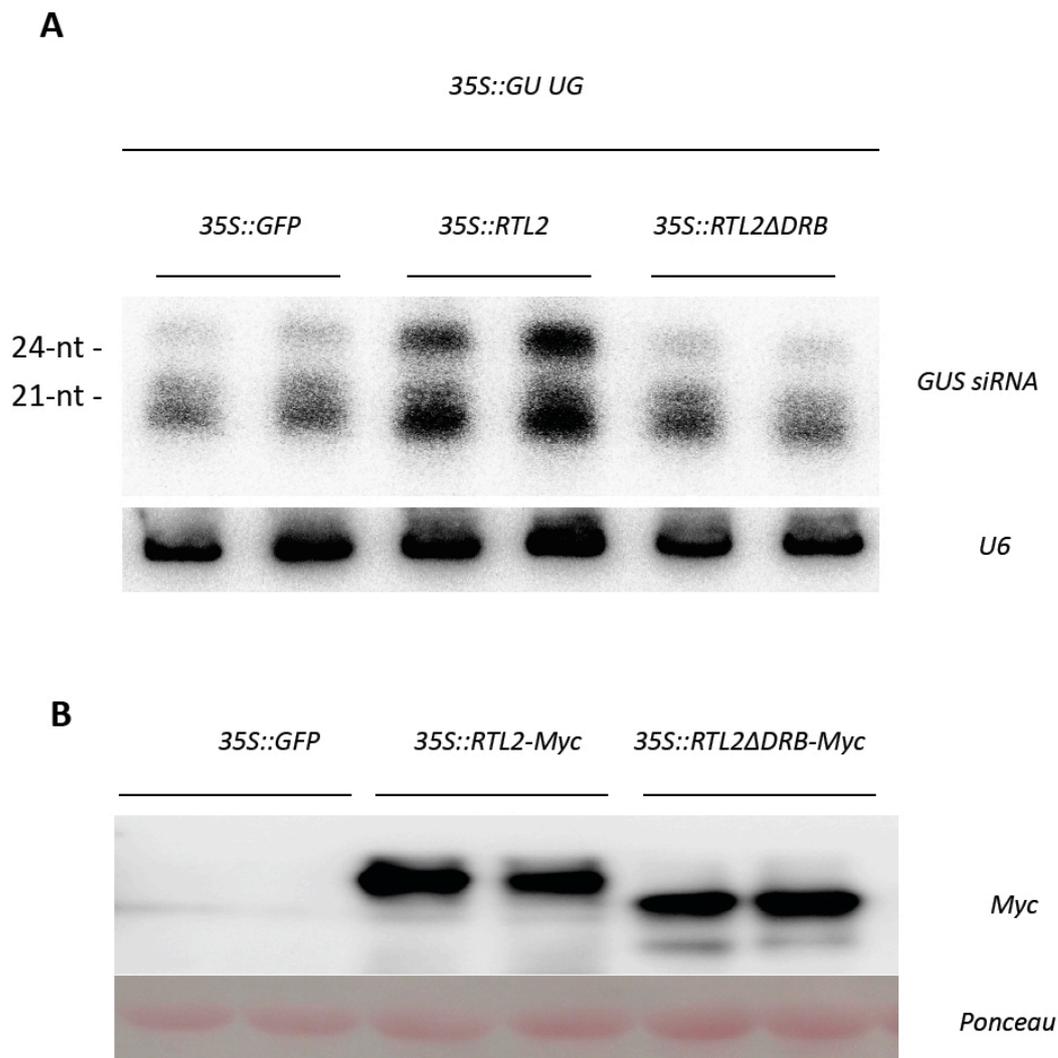
### 3.2.3 - The second DRB domain is required for RTL2 activity

To determine if RTL2 activity requires the presence of functional DRB domains, a construct lacking the most C-terminal DRB domain ( $\Delta$ DRB) was generated. *N. benthamiana* leaves infiltrated with  $35S::GU-UG + 35S::RTL2-\Delta DRB$  accumulated lower level of 21- and 24-nt GU siRNAs, compared with  $35S::GU-UG + 35S::RTL2$ , suggesting that the second DRB domain of RTL2 is required for RTL2 action on both 21- and 24-nt siRNA (Fig.3.8.A). Again, although tagged RTL2 proteins appears inactive, western blot analyses were performed on *N.benthamiana* leaves infiltrated with  $35S::RTL2-Myc$  and  $35S::RTL2-\Delta DRB-Myc$  to determine the effect of the deletion of the second DRB domain on protein stability. Western blot analysis of *N.benthamiana* leaves infiltrated with  $35S::RTL2-Myc$  or  $35S::RTL2-\Delta DRB-Myc$  revealed that the deletion of RTL2 C-terminal DRB domain has no effect on protein levels (Fig.3.8.B), indicating that the second DRB domain is required for RTL2 activity.

### 3.3 – Epistatic relationship between RTL1 and RTL2

Considering the opposite effects of  $35S::RTL1$  and  $35S::RTL2$  on accumulation of GU siRNAs, we analyzed the epistatic relationship between RTL2 and RTL1 by co-infiltrating *N. benthamiana* leaves with  $35S::GU-UG + 35S::RTL1 + 35S::RTL2$  (Fig. 3.9.A). The level of accumulation of GU siRNA was compared with those of  $35S::GU-UG + 35S::GFP$ ,  $35S::GU-UG + 35S::RTL1 + 35S::GFP$  and  $35S::GU-UG + 35S::RTL2 + 35S::GFP$ . The results showed that, for  $35S::GU-UG + 35S::RTL1 + 35S::RTL2$  there was no accumulation of GU siRNA, similar to  $35S::GU-UG + 35S::RTL1 + 35S::GFP$ . We also infiltrated different ratios of  $35S::RTL1$  and/or  $35S::RTL2$ . Irrespective of the different ratios of  $35S::RTL1$  to  $35S::RTL2$ ,  $35S::RTL1$  was found to still be able to inhibit the accumulation of GU siRNA.

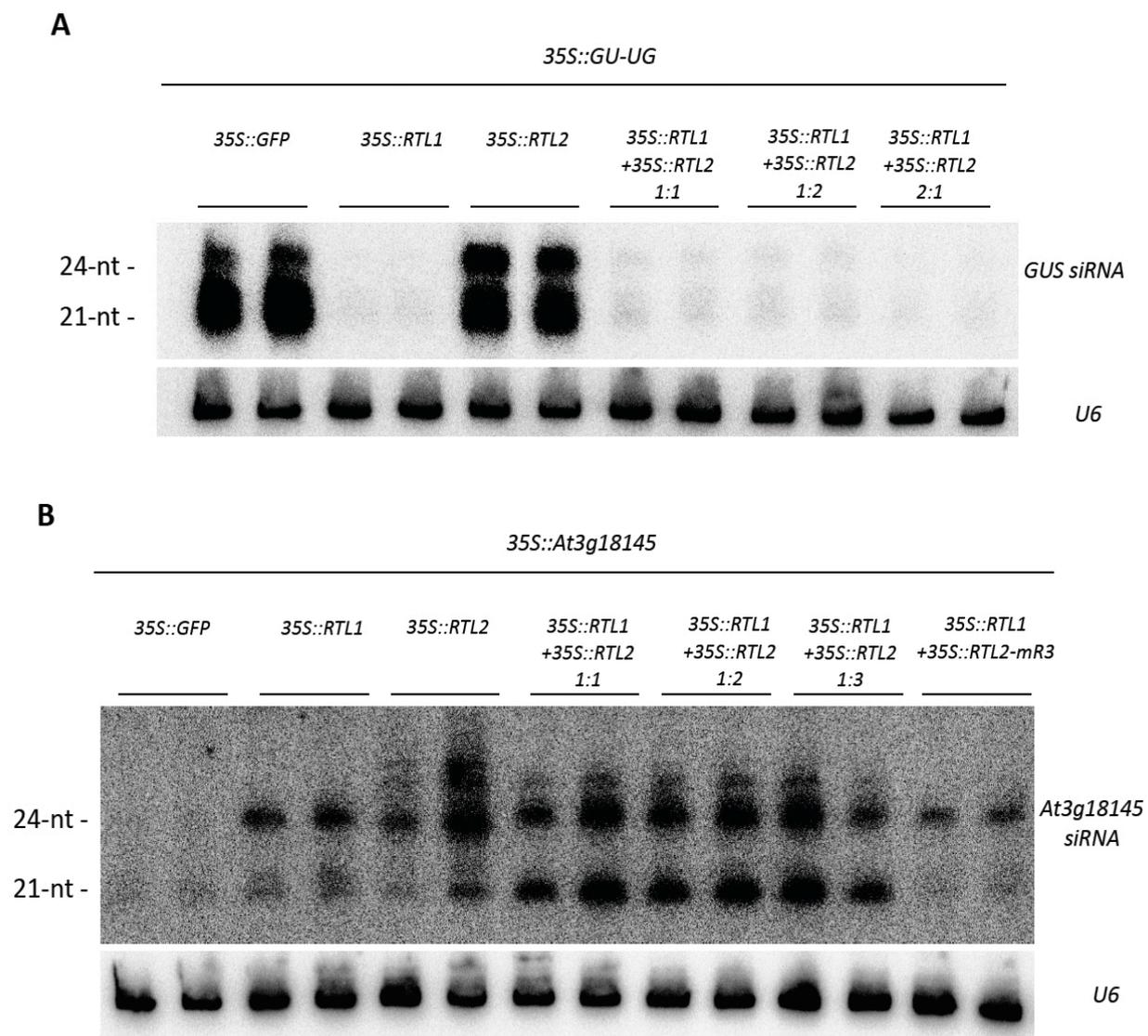
RTL1 over expression can exceptionally result in an over-accumulation of siRNAs at specific loci (for example At3g18145). To confirm the epistatic effect of RTL1 over RTL2 on endogenous targets, we coinfiltrated *N.benthamiana* leaves with  $35S::At3g18145 + 35S::GFP$ ,  $35S::At3g18145 + 35S::RTL1 + 35S::GFP$ ,  $35S::At3g18145 + 35S::RTL2 + 35S::GFP$ ,  $35S::At3g18145 + 35S::RTL1 + 35S::RTL2$  and  $35S::At3g18145 + 35S::RTL1 + 35S::RTL2mR3$  (Fig.3.9.B). As shown in Fig 3.9.B, the coinfiltration of  $35S::At3g18145 + 35S::RTL1 + 35S::GFP$  resulted in the accumulation of 24 nt-siRNAs that are not visible when infiltrating  $35S::At3g18145 + 35S::GFP$ . There was also a slight increase in the amount of 21-nt siRNAs. Interestingly the coinfiltration of  $35S::At3g18145 + 35S::RTL2 + 35S::GFP$  also provoked the accumulation of 24-nt siRNAs and enhanced the accumulation of 21-nt, indicating that RTL1 and RTL2 exceptionally share substrates. However, bands of larger size (likely between 25-nt and 30-nt) were also detected, indicating that they don't have exactly the same activity. For  $35S::At3g18145 + 35S::RTL1 + 35S::RTL2$ , the level of accumulation of 21-nt and 24-nt siRNAs was quite similar to that observed for  $35S::At3g18145 + 35S::RTL2 + 35S::GFP$ , but the amount of bands of higher size was reduced. The level of siRNAs



**Fig 3.8 Effect of the deletion of the second DRB domain of RTL2**

**A)** *N.benthamiana* leaves were infiltrated with the *35S::GU-UG* construct + *35S::RTL2* or *35S::RTL2ΔDRB* or *35S::GFP* as a control. RNA gel blots were hybridized with a GU probe. U6 serves as a loading control.

**B)** *N.benthamiana* leaves were infiltrated with the *35S::GU-UG* construct + *35S::RTL2-Myc* or *35S::RTL2ΔDRB-Myc* or *35S::GFP* as a control. Western blots were hybridized with a Myc probe. Ponceau staining serves as a loading control.



**Fig 3.9 Epistatic relationship between RTL1 and RTL2**

**A)** *N.benthamiana* leaves were infiltrated with the *35S::GU-UG* construct + *35S::RTL1*, *35S::RTL2* or both at the indicated ratio. RNA gel blots were hybridized with a GU probe. U6 serves as a loading control.

**B)** *N.benthamiana* leaves were infiltrated with the *35S::At3g18145* construct + *35S::RTL1*, *35S::RTL2* or both at the indicated ratio. RNA gel blots were hybridized with an oligo probe corresponding to the *At3g18145* 3'UTR-derived 24-nt siRNA detected in Arabidopsis *35S::RTL1* plants. U6 serves as a loading control.

accumulation for coinfiltration of *35S::At3g18145* + *35S::RTL1* + *35S::RTL2mR3*, was similar to those of *35S::At3g18145* + *35S::RTL1* + *35S::GFP*, confirming that RTL2 activity requires an active RNase III domain.

## 4- Discussion

### 4.1 - RTL1

The following reflections are based on the functional studies of two RNase III like enzymes, RTL1 and RTL2. Based on its protein structure RTL1 can be considered as a ClassI RNaseIII member. Its function however is very different from the classical RNA processing role of other members of the RNaseIII family. In fact our results revealed that in *35S::RTL1* plants, there is no accumulation of siRNAs which are usually processed by DCL2, DCL3 or DCL4.

As the overexpression of RTL1 seems to be ineffective on the miRNA population, which is the products of DCL1 processing on imperfectly-paired short hairpins, it is likely that RTL1 can only affect perfectly paired long dsRNA precursors. However RTL1 can interfere with classical DCL1 precursors in specific cases. For example, in wild-type plants, the 3'UTR of the At3g18145 mRNA folds into a long hairpin, and gives rise to very low amounts of 21-nt miR3440b. In contrast, *35S::RTL1* plants accumulate a high amount of a unique 24-nt siRNA molecule, which suggests that RTL1 somehow processes the 3'UTR of the At3g18145 mRNA, thus generating a molecule that can be a substrate for another DCL, which is likely to be DCL3 given that the siRNA is 24-nt in length. Therefore it seems that RNA cleavage activity is more than structure-specific and can also be sequence specific in some rare examples.

Proteins similar to RTL1 can be found in many other plant species. This would seem to prove the importance of RTL1 activity in plants. However under laboratory controlled growth conditions, RTL1 is only very poorly expressed in Arabidopsis roots (Comella et al., 2008). To determine if endogenous RTL1 expression in roots can actually down-regulate siRNA accumulation in this tissue, we compared small RNA sequence level between wild type roots and flowers. The ratio between siRNA and miRNA was lower in roots ( $351415/175233=2.0$ ) compared with flowers ( $11416165/1797097=6.4$ ), which is consistent with the RTL1 siRNA suppressing activity. However, the analysis of *rtl1* mutants will be necessary to sustain this hypothesis.

We also showed that RTL1 could interfere with the accumulation of exogenous siRNAs, such as transgene-derived siRNAs. Therefore RTL1 appears as a general suppressor of plant siRNA pathways. We then wondered if RTL1 could also suppress the accumulation of virus-derived siRNAs and affect the plant anti-viral PTGS defense mechanism.

In fact, PTGS is known to act as an antiviral mechanism in plants, flies and worms. In plants DCL2 and DCL4 are able to process viral dsRNA into siRNAs, which are loaded onto AGO1 or AGO2 to target viral RNAs. However, viruses are also able to interfere with this mechanism by producing viral suppressors of RNA silencing (VSRs). VSRs can interact with PTGS in different steps and they also exhibit different strengths in blocking plant anti viral PTGS defense. For example, the VSR activity of

TYMV p69 is weaker than of CMV 2b, which is weaker than TCV P38. This causes the mild symptoms in TYMV-infected Arabidopsis, intermediate symptoms in CMV-infected and severe symptoms in TCV-infected Arabidopsis.

Our results showed that RTL1 has the potential to counteract the plant antiviral PTGS defense by cleaving dsRNA precursors of viral siRNAs and preventing the loading of virus-derived siRNAs into AGO proteins. Indeed, *35S::RTL1* plants exhibit hyper-susceptibility to infection by TYMV and lack TYMV-derived siRNAs. On the other hand, *35S::RTL1* plants that were infected with TCV, TVCV or CMV, accumulated virus-derived siRNAs suggesting that RTL1 activity is inhibited by these viruses.

In addition infection caused by TCV, TVC and CMV cause similar symptoms on wild-type and *35S::RTL1* plants. It is then possible that the successful infection by these viruses relies on the inhibition of plant antiviral PTGS defense by these viruses and not to RTL1 action.

The fact that TCV, TVCV and CMV strongly inhibit RTL1 activity suggests that the inhibition of RTL1 by certain viruses is essential for other reasons. For example, it may be necessary for certain viruses to inhibit RTL1 activity if RTL1 cleaves their dsRNA replication intermediates, which would impair infection early on. Thus, it is reasonable to assume that viruses that are sensitive to RTL1 at some step(s) of their replication have evolved strategies to inhibit or counteract RTL1 activity. Interestingly, inhibition of antiviral RTL1 activity appears to rely on VSR proteins that also inhibit antiviral plant PTGS defenses. Viral-mediated suppression of RTL1 also could be a strategy to prolong the life of their host plant so that a successful infection can be established before the plant succumbs to death. It will be interesting to determine if viruses also suppress the activity of other known endogenous silencing suppressors.

Our study has revealed a novel plant protein involved in plant-virus interactions. In the case of TYMV, RTL1 serves as an endogenous suppressor of the PTGS defense pathway that may help viruses to infect plants, most likely by degrading dsRNA precursors of virus-derived siRNAs. In fact RTL1 is not the only example of proteins that counteract their antiviral PTGS defense. For example, XRN4 encodes a 5'→3' exoribonuclease that likely degrades viral substrates of PTGS. Indeed, *xrn4* mutants exhibit hyper-resistance to virus infection, indicating that XRN4 counteracts PTGS in wild-type plants (Gy et al., 2007).

However, the inhibition of RTL1 activity by TCV, TVCV and CMV suggests that RTL1 may also serve as an actor of the cellular defenses that help plants to fight against viruses, for example by degrading dsRNA intermediates of virus replication. Further experiments will be required to address the precise action of RTL1 towards each virus.

## 4.2 - RTL2

RTL2 has one RNaseIII and two DRB domains, and thus resemble the atypical Dicer protein from *S.castelli*. RTL2 is found in both dicotyledons and monocotyledonous suggesting evolutionary and functional conservation (Fig. 4.1). AtRTL2 is capable of dimerization (Comella et al., 2008). Most probably, RTL2 homodimers cleave RNAs, as do bacterial RNaseIII and yeast Rtn1 proteins.

Although RTL2 transcripts are detectable in all tissues, no protein is detected in dry seeds. In fact the AtRTL2 protein level decreases during seed formation and becomes detectable after seed imbibition (Comella et al., 2008).

We showed an enhancing effect of RTL2 on the accumulation of exogenous siRNAs. This activity requires a functional RNaseIII domain and a functional second DRB domain.

RTL2 also enhances the accumulation of siRNAs derived from At3g18145, suggesting a possible requirement of RTL2 before DCL action. In animals, Drosha is involved in the process of pri-miRNA to pre-miRNA precursors. Therefore it is possible that, in plants, RTL2 can act in the same way by facilitating the action of DCLs on certain substrates. However, the number of RTL2 substrates likely is limited because *rtl2* mutants do not exhibit morphological defects.

Further in depth studies will be required to complete our understanding of the functional mechanisms of RTL2 in plants.

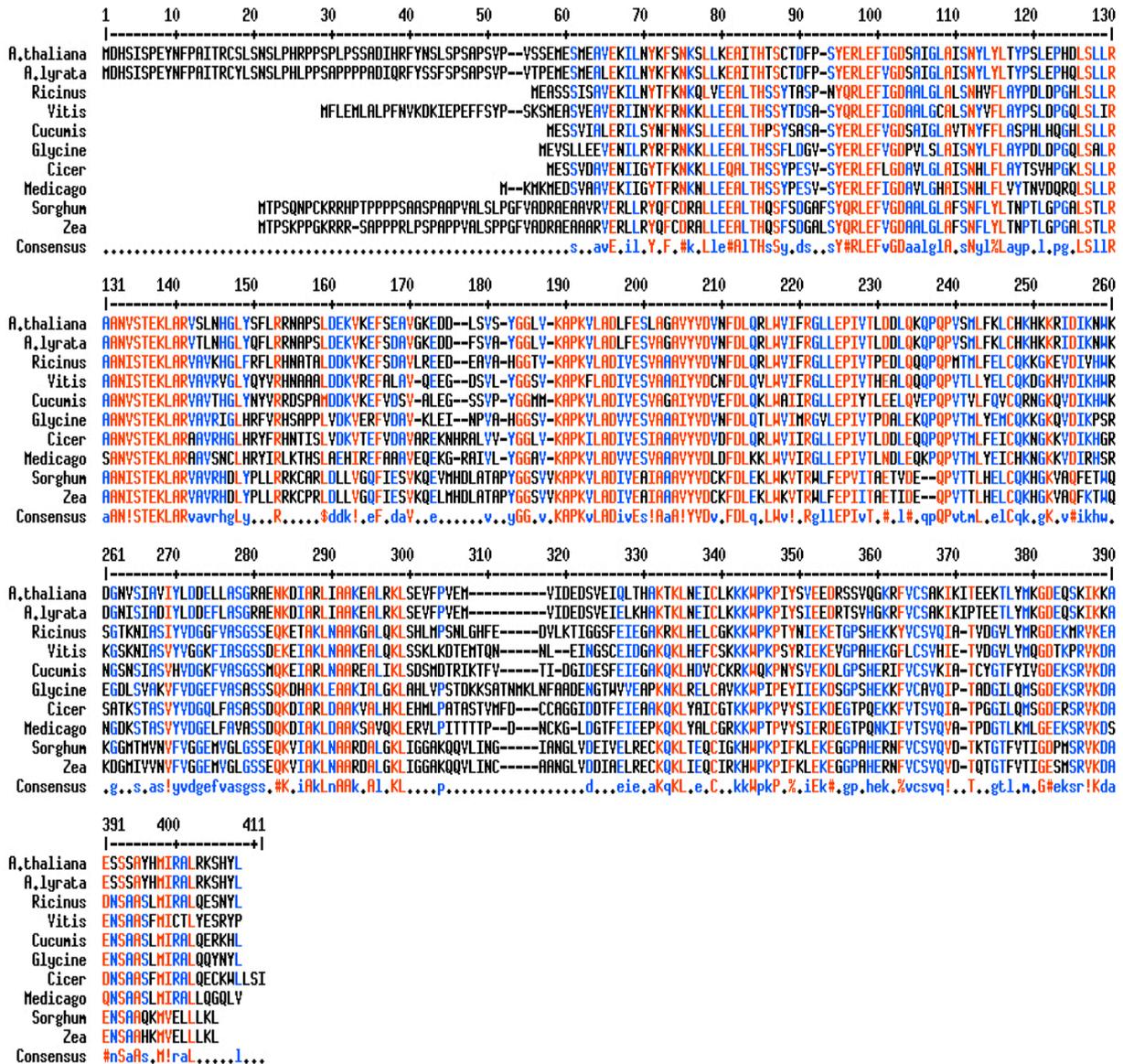


Fig. 4.1: Alignment of plant RTL2 amino acid sequences.

Alignment of RTL1 amino acids sequences from Arabidopsis thaliana ([NP\\_566661.1](#)), Arabidopsis lyrata ([XP\\_002885372.1](#)), Ricinus communis ([XP\\_002529460.1](#)), Cucumis sativus ([XP\\_004147555.1](#)), Vitis vinifera ([XP\\_002278706.1](#)), Glycine max ([XP\\_003544820.1](#)), Cicer arietinum ([XP\\_004491416.1](#)), Medicago truncatula ([XP\\_003602658.1](#)), Sorghum bicolor ([XP\\_002440883.1](#)), Zea mays ([NP\\_001140816.1](#)).

## **5 - Possibilities for future research**

### **5.1 - Short-term goals**

#### **5.1.1 - RTL specificity**

One of the main unanswered questions from this project is the identification of the factors which define RTL specificity. Both RTL1 and RTL2 have very simple protein structures containing one RNaseIII domain and one or two DRB domains. Their RNaseIII motif sequences retain a high degree of similarity and yet they are different enough to enable them to produce the opposite effect on the small RNA accumulation.

The elimination of the RTL2's C-terminal DRB domain, in RTL2 $\Delta$ DRB, did not transform its action to that of RTL1. The number of DRB domains alone therefore is not sufficient for controlling RTL function - other factors must be involved.

In order to further explore this question we plan to perform a swap experiment between RTL1 and RTL2 exchanging their RNaseIII and DRB domains and generating new recombinant RTLswap1-2 and RTLswap2-1 to see if the swap can modify their original activities. At the time of writing, these new recombinant RTLs have already been generated in our lab. Our next step will be to check their effects on the accumulation of different types of small RNAs in *N.benthamiana* and Arabidopsis.

#### **5.1.2 - RTL1 localization**

RTL2 has already been shown to be present in both nucleoplasm and the nucleus (Comella et al., 2008). RTL1 localization however, has not yet been ascertained.

We are therefore performing trials where we are fusing RTL1 to a GFP protein in order to try and pinpoint its location.

#### **5.1.3 - Complimentary viral trails with RTL1**

Although RTL1 expression is induced by viral infections, RTL1 activity is most probably perturbed as a response to VSRs. A series of viral trails are planned using viruses lacking their VSRs on plants with RTL1 overexpression in order to observe the effect of VSRs on RTL1 action and RTL's subsequent effect on plants susceptibility to viral infections.

We have also identified three Arabidopsis lines with overexpression of amiR-RTL1a or amiR-RTL1b, which have low GUS activities in their roots. The effect of viral infection on these three lines will be investigated in near future.

### **5.1.4- RTL2 overexpression in *dcl* mutants**

One of the hypotheses for the enhancing activity of RTL2 on the accumulation of GU siRNAs is that RTL2 interacts with Arabidopsis DCL enzymes. It is possible that RTL2 is involved in the first steps of small RNAs biogenesis and followed by the action of DCLs or, alternatively, it could replace DCL's activity in some places.

To see if this is the case we will overexpress RTL2 in *dcl* mutants and observe its effect on the populations of different types of siRNAs.

## **5.2 - Long term goals**

### **5.2.1 - Functional studies of other RTLs**

The focus of this project was on RTL1 and RTL2 activity only. There are however, three other RTLs to be studied in the future. RTL 4 and RTL5 are present in all plant tissues while RTL3 expression was not detected in any part of the Arabidopsis plants.

*rtl4* mutants are impaired in male and female gametophyte formation and RTL5 is similar to maize RNC1, which splices several chloroplastic group II introns, suggesting essential biological roles for other RTLs. It would therefore be interesting to investigate their respective functions.

### **5.2.2 - RTL interactions with other proteins/RNAs**

RTL proteins have very simple structures and yet very important effects on small RNA populations and plant morphology. It is therefore quite possible that their action comes from their interaction with other proteins or RNAs.

Tagged RTL structures could be used to identify possible co-factors through immunoprecipitation trails.

### **And finally**

This project represents one of first attempts to try to develop a clear picture of RTL functions and their interaction in RNAi pathways. There is still a long way to go and a lot of interesting research to be done!

## 6 - References

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## Résumé

Chez la majorité des eucaryotes, les petits ARN (miRNA et siRNA) jouent des rôles essentiels au cours du développement, dans les réponses adaptatives aux stress, et dans la maintenance de la stabilité génétique. Les plantes codent quatre enzymes RNaseIII de type DICER-LIKE (DCL). DCL1, produit les miRNAs, tandis que DCL2, DCL3 et DCL4 produisent des siRNAs des tailles diverses. Les plantes codent également des enzymes appelées RNASE-THREE-LIKE (RTL) auxquelles il manque certains domaines spécifiques aux DCLs, et dont la fonction est largement inconnue.

Des plantes sur-exprimant RTL1 montrent des défauts morphologiques, et n'accumulent pas les siRNAs produits par DCL2, DCL3 ou DCL4, indiquant que RTL1 est un suppresseur général des voies de siRNA chez les plantes. L'activité de RTL1 nécessite un domaine RNaseIII fonctionnel. RTL1 ne s'exprime naturellement que faiblement dans les racines, mais l'infection virale induite fortement son expression dans les feuilles, ce qui suggère que l'induction de RTL1 est une stratégie générale utilisée par les virus pour contrer la défense antivirale basée sur siRNAs. En accord avec cette hypothèse, les plantes transgéniques sur-exprimant RTL1 sont plus sensibles à l'infection par le TYMV que des plantes de type sauvage, probablement parce que RTL1 empêche la production des siRNAs dirigés contre les RNA viraux. Cependant, les plantes transgéniques sur-exprimant RTL1 ne sont pas plus sensibles à l'infection par le TCV, TVCV ou le CMV, qui codent les suppresseurs de RNA silencing (VSR) plus puissants que le TYMV. En effet, le VSR de TCV inhibe l'activité de RTL1, suggérant que l'induction de l'expression de RTL1 par les virus et l'amortissement de l'activité de RTL1 par leurs VSRs est une double stratégie permettant d'établir une infection avec succès.

Des plantes sur-exprimant RTL2 ou des mutants *rtl2* ne montrent aucun défaut morphologique, et ne montrent pas de changement majeur du répertoire des petits ARNs endogènes. Toutefois, la sur-expression de RTL2 augmente l'accumulation des petits ARNs exogènes dans des essais d'expression transitoire, et cette activité nécessite un domaine RNaseIII fonctionnel. Il est donc possible que RTL2 clive certains substrats pour faciliter l'action des enzymes DCL.