The preferred route for the degradation of silencing target RNAs in transgenic plants depends on pre-established silencing conditions

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ABSTRACT

RNA silencing can be initiated upon dsRNA accumulation and results in homology-dependent degradation of target RNAs mediated by 21–23 nt small interfering RNAs (siRNAs). These small regulatory RNAs can direct RNA degradation via different routes such as the RdRP/Dicer- and the RNA-induced silencing complex (RISC)-catalysed pathways. The relative contribution of both pathways to degradation of target RNAs is not understood. To gain further insight in the process of target selection and degradation, we analysed production of siRNAs characteristic for Dicer-mediated RNA degradation during silencing of mRNAs and chimeric viral RNAs in protoplasts from plants of a transgenic tobacco silencing model line. We show that small RNA accumulation is limited to silencing target regions during steady-state mRNA silencing. For chimeric viral RNAs, siRNA production appears dependent on pre-established cellular silencing conditions. The observed siRNA accumulation profiles imply that silencing of viral target RNAs in pre-silenced protoplasts occurs mainly via a RISC-mediated pathway, guided by (pre-existing) siRNAs derived from cellular mRNAs. In cells that are not silenced at the time of infection, viral RNA degradation seems to involve Dicer action directly on the viral RNAs. This suggests that the silencing mechanism flexibly deploys different components of the RNA degradation machinery in function of the prevailing silencing status.

INTRODUCTION

RNA silencing in many eukaryotes has been shown to be intimately linked to production of small interfering RNAs

(siRNAs). It has been demonstrated that siRNAs can be generated as small duplex molecules through processing of dsRNA trigger molecules (1,2). This processing step is catalysed by an RNase-III-like enzyme referred to as Dicer (1). siRNAs were shown to mediate degradation of silencing target RNAs (3–5) by guiding an RNA-induced silencing complex (RISC) to homologous target RNAs for endonucleolytic cleavage (6). Further data suggest that siRNAs are also able to mediate their own amplification. Observations made in *Caenorhabditis elegans* and plants indicate that siRNAs can serve as primers for the synthesis of dsRNA on ssRNA templates by RNA-dependent RNA polymerases (7–12). The resulting dsRNA is thought to be processed by Dicer resulting in production of secondary siRNAs. This can lead to spreading of silencing along the template and to new templates, a phenomenon referred to as transitive silencing (10). In summary, the data indicate that silencing target RNAs can be degraded via multiple routes. In all cases, silencing initiation involves Dicer-mediated cleavage of dsRNA targets. Degradation of secondary targets may again involve Dicer activity on these targets, or may be completely dependent on RISC action, guided by siRNAs derived from the primary targets.

The relative importance of the siRNA-guided RISC and RdRP/Dicer pathways for target RNA degradation appears to differ between organisms. In *Drosophila* and mammals, no RdRP homologues have been found. Accordingly, no transitivity or siRNA amplification mechanism has been observed in cultured cells or intact organisms (13,14), despite an earlier observation in a *Drosophila* cell-free system that suggested the existence of such a mechanism (15). In plants and *C.elegans*, RdRP activities have been demonstrated and mutant phenotypes indicate a role for RdRP in RNA silencing in these organisms (16,17). However, the absence of transitive silencing in several plant silencing cases (11,18,19) suggests that these RdRP-mediated pathways are not always activated. Furthermore, these observations suggest the existence of a regulation mechanism that controls the activity of these pathways.

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The key factors responsible for the regulation of the different RNA degradation pathways are not known. Selective entry of a target RNA into a specific degradation pathway could be dependent on the phase of silencing (initiation, maintenance, systemic spread), on the nature of the target itself (e.g. aberrant versus 'normal' mRNA, viral versus cellular RNA) or could even differ for sequences within the same template due to varying intrinsic properties such as primary sequence or secondary structure.

In this study we investigated which silencing-related RNA degradation pathways are active during silencing of mRNAs and chimeric viral RNAs in protoplasts from silenced and non-silenced plants. For this purpose we made use of the transgenic tobacco line T17, which is a model for glucanase gene silencing (20,21). T17 plants homozygous for the *Nicotiana plumbaginifolia gnl* transgene display co-suppression of the transgene and the homologous endogenous genes whereas hemizygous T17 plants do not exhibit co-suppression. Selection of different silencing pathways was monitored via quantification of the accumulation levels of mature mRNAs and viral RNAs and siRNAs corresponding to various regions of the mature RNAs. We previously demonstrated that protoplasts are particularly suited to study early steps of RNA silencing (20,21), since they allow accurate quantification of RNA levels and there is no interference of silencing processes not directly related to target RNA degradation such as systemic spread of silencing.

We show here that small RNAs corresponding to mRNA target regions accumulate in silenced T17 plants whereas no small RNAs corresponding to non-target regions could be detected. Importantly, we observed that, upon infection with chimeric viral RNA, virus-derived small RNAs accumulate in protoplasts of non-silenced, *gnl* expressing plants, suggesting induction of a silencing-like process at the single cell level. In contrast, in pre-silenced protoplasts, accumulation of chimeric viral RNAs was strongly suppressed without leading to a detectable accumulation of virus-specific small RNAs. These data imply that silencing of chimeric viral RNAs in pre-silenced protoplasts occurs predominantly via a RISC-mediated pathway, guided by mRNA-derived siRNAs, whereas in *gnl* expressing protoplasts chimeric viral RNA degradation involves Dicer activity directly on the viral RNA. This implies that the silencing mechanism is flexible with respect to how it deals with specific silencing targets under various conditions.

MATERIALS AND METHODS

Plant material and growth conditions

Homozygous and hemizygous T17 plants [transgenic *Nicotiana tabacum* cv Petit Havana SR1 (20)] and untransformed SR1 tobacco plants were germinated and grown *in vitro* on solid medium in a growth chamber (25°C, 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 14 h light/10 h dark period). Plants were propagated via cuttings every 7 to 9 weeks.

Plasmid constructions

The construction of plasmid pTNV-A containing a full-length cDNA copy of the TNV-A and permitting synthesis of

infectious *in vitro* transcripts, is described elsewhere (22). The construction of plasmid pSTNV-L is described in chapter 2.

In vitro transcription of viral RNAs

TNV-A RNA was synthesized *in vitro* from plasmid pTNV-A linearized at the BsaI site, using T7 RNA polymerase. Chimeric STNV-L RNAs were synthesized *in vitro* from the appropriate pSTNV plasmids linearized at the BamHI site, using T7 RNA polymerase. In chimeric STNV-L RNA, the 5' terminal A residue is replaced by a G residue, and a 3' terminal extension of 7 nt is added to the trailer, relative to wild-type STNV-2. All *in vitro* transcripts were prepared using the Megascript kit (Ambion). The final RNA concentration was determined spectrophotometrically and the integrity and length of all transcripts was assessed by denaturing agarose gel electrophoresis of denatured RNA samples.

Protoplast preparation and electroporation experiments

Protoplasts were prepared from the top leaves of 7- to 9-weeks-old plants grown from cuttings, as described by De Block *et al.* (23). Closely spaced parallel incisions were made in leaves, which were subsequently placed in Petri dishes of 9 cm diameter containing 13 ml incubation medium to which cellulase (0.75%) and macerace (0.3%) were added (leaves face up, covering the entire surface of the dish once). The dishes were incubated for 18 h at 25°C in the dark. Undigested material was removed by sieving through 200 and 100 μm mesh sieves. Protoplasts were purified and concentrated by repeated flotation centrifugations in fresh incubation medium at 80 g.

Protoplasts were electroporated as described by Meulewaeter *et al.* (24). For 1×10^6 protoplasts, 0.2 pmol TNV-A RNA and 2 pmol chimeric STNV-2 RNA were used as an inoculum. In one experiment (Figure 4), 0.5 pmol TNV-A RNA and 5 pmol chimeric STNV-L RNA were used per 10^6 protoplasts. After the electroporation, the protoplasts were washed in order to remove dead cells and excess, extracellular inoculum RNA. Subsequently, the protoplasts were incubated at a concentration of 0.5 to $1 \times 10^6 \text{ ml}^{-1}$ in 5 ml incubation medium, in the dark at 24°C. At the time points indicated in the experiments, dead cells were removed by centrifugation at 80 g, and RNA was extracted from the surviving (floating) protoplasts.

Small RNA isolation and analysis

To detect small RNA species, an adapted version of the Hamilton and Baulcombe (25) protocol was used. Total nucleic acids were extracted from protoplasts of expressing and silenced T17 plants. From the nucleic acid extract, low molecular weight RNA was enriched by precipitation with 10% polyethylene glycol (MW 8000) and 0.5 M NaCl. The low molecular weight fraction was separated by electrophoresis through 15% polyacrylamide-7 M urea-0.5 $\times$ tris borate EDTA gels, transferred onto Hybond N+ filters (Amersham) and fixed by ultraviolet cross-linking. The filters were prehybridized in 45% formamide, 7% SDS, 0.3 M NaCl, 0.05 M $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ (pH 7), 1 $\times$ Denhardt's solution and denatured herring sperm DNA (100 mg/ml). Hybridization was in the same solution using hydrolyzed α - ^{32}P -labelled RNA probes with an average length of 50 nt corresponding to the (+) or (−) strand of nearly the entire *gnl* mRNA region

or sub-regions K, L, D, Up-L or Down-L of the *gnl* mRNA and viral STNV-L RNA. Hybridized filters were washed two times for 20 min with 2× SSC/0.2% SDS at 50°C. PCR fragments containing the relevant sequences linked to a T7 (sense transcripts) or a SP6 (antisense transcripts) promoter were used to generate the riboprobes. Control hybridizations on Southern blots showed that the riboprobes were specific for the selected region.

For the reversed hybridizations, small nucleic acids were cut from a ethidium-bromide-stained 15% polyacrylamide–7 M urea–0.5 × tris borate EDTA gel containing RNA size markers. The relevant gel pieces were crushed and eluted in 2 vol elution buffer (80% formamide, 40 mM Pipes pH = 6.4, 1 mM EDTA, 400 mM NaCl) at room temperature overnight with gentle shaking. The eluted RNA was precipitated with 2 vol of 96% ethanol by centrifugation, washed with 70% ethanol and resuspended in nuclease free water. Half of the material was used for dephosphorylation by alkaline phosphatase (CIP). The dephosphorylated and untreated fraction were end-labelled by T4 polynucleotide kinase in the presence of [γ -³²P]ATP. The labelled probes were used on Southern blots containing various sequences the *gnl* and *glb* mRNA region.

RESULTS

Co-suppressed endogenous and transgenic glucanase genes show a similar distribution of target and non-target sequences in their mRNAs

We have previously shown that glucanase mRNA silencing in T17 plants leads to accumulation of 5' and 3' *gnl* mRNA degradation intermediates (26). We further showed that RNA silencing in T17 plants is active against mRNA-internal but not against 5' and 3' terminal regions of *gnl* mRNAs (21). Finally, we observed siRNAs for internal regions of *gnl* mRNAs. Taken together, these data are consistent with the idea that silencing-related *gnl* mRNA degradation in T17 plants mainly proceeds via RISC-catalysed endonucleolytic cleavage of internal mRNA regions, guided by siRNAs corresponding to these same regions. Our analysis could however not exclude that terminal *gnl* sequences in their natural mRNA context are targeted via the RdRP/Dicer pathway for siRNA production but protected from RISC-mediated degradation. This latter scenario would predict the presence of siRNAs for 'non-target' regions and would imply that RdRP/Dicer and RISC have differential access to *gnl* target sequences. To investigate this, we explored the relationship between siRNA production and silencing target efficiency.

Since the route of target RNA degradation pathways could be different for transgenic and endogenous mRNAs, we investigated in a first experiment whether the same distribution of target (internal) and non-target (terminal) sequences exists for the co-suppressed endogenous mRNAs as for the transgenic mRNAs. Through the use of a dual viral reporter system (21), we examined targeting of internal and terminal regions of a co-suppressed endogenous glucanase gene and compared the silencing efficiencies with those of corresponding transgene mRNA regions (Figure 1A and B). The *glb* gene was chosen as a representative for the family of highly homologous co-suppressed endogenous β -1,3-glucanase genes in *N.tabacum*.

Chimeric STNV RNAs containing corresponding *gnl* and *glb* regions (Lex, K, L and T; Figure 1A) were co-delivered with TNV RNA into protoplasts of hemizygous, glucanase-expressing (He) and homozygous, silenced T17 plants (Ho). Twenty hours after delivery chimeric STNV RNA levels were measured. The relative accumulation level of chimeric STNV RNA in protoplasts of hemizygous compared to homozygous plants (He/Ho ratio) was taken as a measure for the silencing susceptibility of the sequence inserted in the chimeric STNV RNA. To verify whether differences in chimeric STNV–glucanase RNA accumulation in electroporated protoplasts were caused by variation in replicase availability, TNV RNA accumulation was systematically measured in the inoculated protoplasts. In all samples, TNV accumulated to comparable levels implying that observed differences in STNV–glucanase RNA accumulation were due to different efficiencies of silencing (Figure 1C).

In agreement with previous observations, chimeric STNV RNAs that contained internal mRNA regions of both glucanase genes accumulated to lower levels in protoplasts of silenced compared to expressing plants, indicating these regions are targets for silencing. In contrast, chimeric STNV RNAs containing 5' and 3' terminal sequences of both glucanase mRNAs (regions eLex, tLex, tT, eT in Figure 1A) accumulated to similar levels in protoplast of silenced and expressing plants, implying that termini of both glucanase mRNAs are not recognized by the silencing machinery. Taken together, the data show that, though silencing target efficiencies differ in quantitative terms (Figure 1D), the distribution of target and non-target sequences is the same for the co-suppressed endogenous *glb* gene and the *gnl* transgene and silencing in the T17 line is primarily directed against internal regions of both co-suppressed glucanase mRNAs.

Silenced T17 plants accumulate small 21–23 nt RNAs corresponding to silencing target regions

In the next step, we examined to what extent the 21–23 nt RNAs accumulating in T17 plants correspond to target and non-target regions of the *gnl* mRNA. We have previously shown that small 21–23 nt glucanase RNAs accumulate in silenced T17 plants (9). However, in these experiments we did not discriminate between target and non-target regions. In the current approach, we used region- and strand-specific riboprobes to detect siRNAs corresponding to 5' and 3' terminal non-target regions of the *gnl* mRNA and corresponding to an internal target region of *gnl*. Three probes for plus-strand detection were hybridized against identical membranes carrying low molecular weight nucleic acid fractions from leaf protoplasts of silenced (Ho) and expressing (He) T17 plants (Figure 2).

Control hybridization with a nearly full-length *gnl* mRNA riboprobe yielded small RNA signals of similar intensity on all three membranes, indicating that in each case the low molecular weight RNAs were blotted equally efficiently (data not shown). Hybridization with the region-specific probes showed that small-sense RNAs could be detected with the internal (Δ O) riboprobe, but not with the 5' and 3' (tLex and tT) riboprobes. Similar results were obtained for minus-strand detection (data not shown). This indicates that small RNAs of both polarities corresponding to target but not to non-target regions accumulate in silenced T17 plants.

Figure 1. Silencing is primarily directed against internal mRNA regions of the *gnl* transgene and a co-suppressed glucanase gene (*glb*). (A) Schematic presentation of the *gnl* and *glb* mRNAs. Exons (Ex), leaders (Le) and 3'-untranslated regions (3'-UTRs) are indicated. The *glb* test regions (eLex, eK, eL and eT) and the corresponding *gnl* test regions (tLex, tK, tL and tT) are represented with solid lines. Plasmids carrying the test regions between the STNV leader and trailer (22) were linearized and *in vitro* transcribed to produce chimeric viral RNAs for delivery into protoplasts. (B) Features of the test regions Lex, K, L and T: The length of these test sequences is shown, as well as the overall homology between endogenous and transgenic sequences. (C) Northern blot analysis of total RNA extracted from protoplasts of hemizygous expressing (He) and homozygous silenced (Ho) T17 plants 20 h after delivery of TNV RNA and chimeric STNV RNA containing transgenic and endogenous test sequences as shown in (A). ³²P-labelled RNA probes for detection of viral RNAs and ribosomal RNA were complementary to the (+) strand of the STNV trailer, the (+) strand of TNV and 18S rRNA sequences. (D) Relative accumulation of chimeric STNV RNAs in protoplasts of hemizygous versus homozygous T17 plants (He/Ho ratio). Bars represent the average of at least two independent experiments.

To investigate the relationship between siRNA accumulation and silencing target selection in more detail, we performed reverse northern blot hybridizations in which small RNAs extracted from protoplasts of silenced T17 plants (see Materials and Methods) were used as a probe against a panel of transgene- (*gnl*) and endogene-derived (*glb*) DNA sequences. This set up allows direct scanning of sequences throughout the silencing target mRNAs for small RNA production in a single hybridization. Although the DNA fragments on the membrane included both *gnl*- and *glb*-specific sequences, we did not expect the small RNA probes to distinguish between transgenic and endogenous internal mRNA sequences as the nucleotide sequence homology between

gnl and *glb* in these internal regions is relatively high (78–83%).

The small RNAs accumulating in protoplasts of silenced T17 plants were ³²P-end-labelled using T4 polynucleotide kinase. As we do not know the phosphorylation status of the small RNAs that accumulate in silenced T17 plants we opted to use half of the isolated small RNAs for direct 5' end labelling and half to be dephosphorylated prior to end labelling (see Materials and Methods). Both types of small RNA probes were used on separate, identical blots. Measurement of the radioactivity in the probes and analysis of the autoradiograms showed that end labelling of dephosphorylated small RNAs was much more efficient compared to end labelling of

Figure 2. Small RNAs corresponding to an internal target sequence but not to proximal and distal ends of the *gn1* mRNA accumulate in protoplasts of silenced T17 plants. (A) Schematic presentation of the length and position of probe templates in relation to the *gn1* mRNA. Exons are indicated. Le: leader, 3'-UTR: 3'-untranslated region. The probe regions are represented with solid lines. (B) Northern blot analysis of low molecular weight RNA (30 μg) from protoplasts of hemizygous, expressing (He) and homozygous, silenced (Ho) T17 plants using ^{32}P -labelled RNA probes corresponding to the (–) strand of the Lex2, ΔO and T region in the *gn1* mRNA. The arrow indicates the position of the small RNA species. Lanes designated C contain *in vitro* synthesized RNA complementary to the probe used and ssRNA size markers (20 and 25 nt). RNA size markers on all filters were detected through rehybridization with ^{32}P -labelled RNA probes corresponding to nearly the entire *gn1* mRNA, showing that detected small RNAs are within the 20–25 nt size range (data not shown).

untreated small RNAs (data not shown). This suggests that the majority of small RNAs accumulating in silenced T17 plants are 5' phosphorylated.

Analysis of the membrane hybridized with the small RNA probe that was dephosphorylated prior to end labelling, shows that DNA fragments corresponding to internal regions of the *gn1* and *glb* mRNA, which are efficient silencing targets, are detected (Figure 3: regions tK, tL, eK and eL). In contrast, the fragments corresponding to the non-targeted proximal and distal ends of the *gn1* and *glb* mRNAs were not detected (Figure 3: eLex, eT, tLex, tT). A very weak signal was obtained for the fragment corresponding to the tD and tJ regions of *gn1*. For the tD region this is consistent with this region being a relatively inefficient target. The tJ region was previously shown to be a good silencing target. The result observed here suggests that only a small region within tJ is a source for siRNA production.

The results obtained via the forward and reverse northern hybridization experiments consistently showed that the small RNAs accumulating in silenced T17 plants correspond to silencing target regions and not to non-target regions of the co-suppressed glucanase mRNAs. The data suggest that non-target regions are no template for siRNA synthesis.

Small RNAs are produced in non-silenced cells upon accumulation of high amounts of chimeric viral RNA

While the silencing mechanism appears competent to discriminate between target and non-target regions within co-suppressed mRNAs, it is not clear how elected targets are addressed by different components of the silencing machinery under different silencing conditions. For example, the relative contribution of siRNA-guided RISC- and RdRP/Dicer-mediated pathways to target degradation during established silencing conditions and during the onset of silencing is not understood. To investigate this we introduced viral RNAs as silencing targets into non-silenced and pre-silenced cells and measured the accumulation of viral RNA and virus-derived siRNAs. To further understand the interplay between silencing of viral RNAs and mRNAs, we also analysed the impact of viral RNA accumulation and silencing on the silencing activity targeted against homologous cellular RNAs.

In a first set of experiments we delivered chimeric viral RNA containing a silencing target region (L region of *gn1*; STNV-L RNA; Figure 4.I.) together with TNV RNA to protoplasts of non-silenced *gn1* expressing T17 plants and examined small RNA accumulation twenty hours after co-delivery. In agreement with previous experiments we observed that chimeric viral RNA accumulates to high levels in non-silenced T17 protoplasts (data not shown). Analysis of the low molecular weight RNA fraction clearly showed that small RNAs corresponding to the L region of *gn1* accumulate in virus-inoculated protoplast of *gn1* expressing plants (Figure 4.II.). No small RNAs were detected in non-inoculated protoplasts of expressing plants. This implies the induction of a silencing-like process in these protoplasts as a result of the accumulation of high amounts of chimeric viral RNA.

The small RNAs corresponding to the L region observed in virus-infected, non-silenced protoplasts could be directly produced from the viral dsRNA if this is a Dicer substrate. Alternatively, these small RNAs could originate from the *gn1* mRNA, in case viral RNA accumulation triggers initiation of a silencing process targeted towards the *gn1* mRNA. To understand the origin of siRNAs in protoplasts of non-silenced plants inoculated with viral RNA we further characterized the nature of the accumulating small RNAs by using region-specific riboprobes corresponding to leader and trailer sequences of the STNV RNA (Figure 4.I.) and probes for specific regions of the *gn1* mRNA not present in STNV-L (Figure 4.I.). Figure 4.II.B shows that no small RNAs corresponding to *gn1* specific regions were detected. In contrast, small sense and anti-sense RNAs corresponding to STNV leader and trailer sequences were detected in infected protoplasts of expressing T17 plants (Figure 4.II.C). These results indicate that the small RNAs accumulating in infected protoplasts of expressing T17 plants are predominantly derived from the virus and that, within the time frame of the experiment, there is no discernable cross-talk to the mRNA in terms of siRNA production.

Even in the absence of mRNA-derived siRNAs, the virus-derived small RNAs could mediate degradation of the *gn1* mRNA in the region of sequence homology (L region). To investigate this, we compared the *gn1* mRNA accumulation levels in infected and non-infected protoplasts of expressing

Figure 3. Small RNAs accumulating in protoplasts of silenced T17 plants correspond to target but not to non-target glucanase mRNA regions. (A) Schematic presentation of the *gnI* (tLex, tJ, tK, tL, tD, ΔP69 and tT) and *glb* (eLex, eK, eL and eT) test regions. Exons are indicated. Le: leader, 3'-UTR: 3'-untranslated region. (B) Ethidium-bromide-stained 2% agarose gel containing a panel of DNA sequences corresponding to different regions in the *gnI* and *glb* mRNA. ΔP69 is a ssDNA oligonucleotide of 81 nt consisting of a 69 nt sequence corresponding to a sense sequence fragment within the L region and 12 nt of *gnI*-unrelated sequence. (C) Phosphor image of the gel shown in (B) after blotting and hybridization with the labelled small RNA from protoplasts of silenced T17 plants. Signals indicate the presence of siRNAs for corresponding regions.

T17 plants. We did not detect significant differences in *gnI* mRNA levels (data available as supplementary information), indicating that no substantial silencing of the *gnI* mRNA occurred in infected protoplasts of expressing plants within the timeframe of the experiment.

Taken together, the results imply that in virus-infected protoplasts of hemizygous, glucanase expressing T17 plants production of virus-derived siRNAs is initiated. Presumably this involves Dicer-like activity and results in attenuated accumulation of the viral RNAs. Importantly, there is no significant feedback towards the homologous transgene in terms of siRNA production and mRNA degradation within the time-frame of the experiment.

Accumulation of viral silencing target RNAs does not affect the abundance of small RNAs accumulating in protoplast of silenced plants

The previous experiments showed that a Dicer-like activity can be activated in non-silenced T17 cells, upon introduction of chimeric STNV RNA. This leads to production of virus-derived siRNAs and, presumably, to attenuated accumulation of viral RNA. It is unclear how chimeric viral RNAs with homology to a silencing target sequence are targeted in a pre-established silencing situation, with silencing factors already being activated.

To address this question we monitored siRNA production upon viral RNA inoculation in pre-silenced cells. To this end, TNV RNA together with STNV-L RNA was delivered to

protoplasts of silenced T17 plants and the accumulation of mature viral RNA and small RNA was measured 20 h after delivery. As previously observed, and consistent with the L-region being a silencing target, chimeric STNV-L accumulation in protoplasts of silenced plants was strongly reduced compared to non-silenced plants (data not shown). Small RNAs corresponding to region L accumulated to similar levels in inoculated and non-inoculated protoplasts of silenced plants (Figure 5A), suggesting that viral STNV-L RNA does not contribute to the pool of L-derived siRNAs, or the level of L-derived siRNA is at a plateau. Importantly, no small RNAs corresponding to the virus-specific region upstream of the L sequence was detected (Figure 5B). On the same membrane, these virus-specific small RNAs were detected in the low molecular weight nucleic acid fraction extracted from virus-inoculated protoplasts of *gnI*-expressing (He) T17 plants. Together, these data suggest that, within the time-frame of the experiment, no *de novo* synthesis of virus-derived siRNAs occurred in virus-inoculated pre-silenced cells, while at the same time a silencing mechanism targeting the chimeric viral RNA is active in these cells. We conclude that this silencing activity is RISC-based and guided by mRNA-derived siRNAs that were already available at the time-point of infection.

Infection of pre-silenced cells with viral RNAs and subsequent diversion of siRNA-programmed RISC complexes to the novel targets may lead to an altered silencing efficiency for the originally targeted mRNAs. To investigate this, we compared the *gnI* mRNA levels in infected and

I.

II.

Figure 4. Small virus-derived RNAs accumulate in virus-infected protoplasts of non-silenced T17 plants. (I) Schematic presentation of probe regions in relation to the *gnl* mRNA and STNV-L RNA. The probe regions are represented with solid lines. Exons are indicated. (II) Low molecular weight nucleic acid fractions from protoplasts electroporated with TNV RNA and STNV-L RNA (0.5 pmol and 5 pmol per 10^6 protoplasts, respectively) and from non-electroporated protoplasts of hemizygous, expressing T17 plants were separated on a 15% polyacrylamide gel (20 μ g) and blotted to membranes. (A) Filters hybridized with 32 P-labelled RNA probes corresponding to the (+) strand of the L region in the *gnl* mRNA and chimeric viral RNA. (B) Filters hybridized with 32 P-labelled RNA probes corresponding to the (+) and (–) strand of the D and K region in the *gnl* mRNA. (C) Filters hybridized with 32 P-labelled RNA probes corresponding to the (+) and/or (–) strand of the Up-L and Down-L regions in the viral RNA. The arrows at the left side of the blots indicate the position of the small RNA species. 20, 22 and 25 nt size markers are presented at the right side of the blots. The presented phosphor images were obtained from equal exposures to phosphor imager screens. nep: low molecular weight nucleic acid fraction from non-electroporated protoplasts of non-silenced plants; 44 h.: low molecular weight nucleic acid fraction from (TNV and STNV-L) electroporated protoplasts of non-silenced plants 44 h post-electroporation.

non-infected protoplasts of silenced T17 plants. Due to the low accumulation levels of *gnl* mRNA in both infected and non-infected protoplasts of silenced T17 plants (data not shown), it was impossible to evaluate whether mRNA silencing was further enhanced through introduction of viral target RNA. However, the results did indicate that mRNA silencing efficiency was at least not drastically reduced by the viral RNA infection within the timeframe of the experiment. This implies that the silencing machinery was not saturated by the introduction of high amounts of additional silencing target RNA.

Taken together these data show that introduction into silenced T17 cells of high amounts of viral RNA that is targeted by the silencing machinery does not significantly influence the accumulation of transgene-derived siRNAs and does not lead to detectable accumulation of virus-specific siRNAs. This strongly suggests that degradation of chimeric viral target RNAs in silenced cells is driven by cellular pre-existing siRNAs and executed by a RISC-like activity. In conjunction with the results obtained in expressing cells, the data suggest that in terms of small RNA synthesis pre-silenced and *gnl*-expressing cells react differently to virus infection.

Figure 5. Virus inoculation results in differential production of virus-specific RNAs in protoplasts of silenced and non-silenced T17 plants. Low molecular weight nucleic acid fractions (15 μ g) from protoplasts electroporated with TNV RNA and STNV-L RNA (0.2 pmol and 2 pmol per 10^6 protoplasts, respectively) and from non-electroporated protoplasts of hemizygous, expressing (He) and homozygous silenced (Ho) T17 plants were separated on a 15% polyacrylamide gel and blotted to membranes. (A) Filters hybridized with 32 P-labelled RNA probes corresponding to the (+) or (–) strand of the L region in the *gnl* mRNA. (B) Filters hybridized with 32 P-labelled RNA probes complementary to the (+) strand of the Up-L region in the viral STNV-L RNA. The arrows indicate the position of the small RNA species. Comparison to RNA size markers indicates that the small RNA species are in the 20–25 nt range. nep: low molecular weight nucleic acid fraction from non-electroporated protoplasts of silenced and non-silenced plants; 20 h.: low molecular weight nucleic acid fraction from (TNV and STNV-L) electroporated protoplasts from silenced and non-silenced plants 20 h post-electroporation.

DISCUSSION

In this study we have analysed the relationship between silencing target selection and siRNA accumulation for the co-suppressed glucanase mRNAs. Furthermore, we have studied the impact of chimeric viral RNA accumulation on siRNA production and silencing kinetics in silenced and non-silenced cells. We show that siRNAs corresponding to internal glucanase mRNA target regions accumulate in protoplasts of silenced T17 plants whereas no siRNAs were detected for the non-target proximal and distal mRNA termini. We also show that introduction of chimeric viral target RNAs in pre-silenced protoplasts results in silencing of these viral RNAs without causing the accumulation of detectable amounts of virus-specific siRNAs, nor enhancing the accumulation of glucanase-specific siRNAs, derived from sequences that are shared between the chimeric virus and the silenced transgene. In contrast, introduction of chimeric viral RNAs in non-silenced protoplasts does result in production of virus-derived siRNAs, without leading to a clearly detectable silencing effect directed against the chimeric viral RNA or *gnl* mRNA. These results point towards a regulation mechanism that controls the activity of silencing factors in response to the context of the target sequence and in function of the pre-established silencing situation.

The absence of small RNAs corresponding to proximal and distal non-target regions of the glucanase mRNAs while being present for internal transgene- and endogene-specific glucanase mRNA regions indicates that terminal mRNA sequences

are not involved in siRNA production. This rules out the possibility that for non-target regions siRNAs are produced that fail to guide RISC-mediated RNA degradation. The results also imply that certain (proximal and distal) sequences within a silencing target can be protected from the spreading mechanism underlying transitive silencing. The factors that control this differential silencing activity *in cis* have yet to be identified.

The accumulation of chimeric viral RNA in protoplasts of non-silenced plants resulted in the production of small RNAs, suggesting the induction of a silencing-like activity in single cells. However, this did not lead to observable effects for the homologous transgene in terms of mRNA abundance and siRNA production. The absence of any silencing effect of virus-derived siRNAs on homologous mRNAs could be due to several factors. Protoplasts from T17 plants might not be competent to induce mRNA silencing at the single cell level or the set-up of mRNA silencing could require a time period for induction of silencing activities (RdRP or RISC activation, for instance) that is longer than the time-span of the experiments (44 h). A specific limitation could be that the small virus-derived siRNAs produced in T17 protoplasts lack the ability to guide RISC-mediated silencing of glucanase mRNA targets or to prime RdRP-dependent siRNA amplification. This could be if, e.g., initiation of mRNA silencing required a nuclear step the virus is unable to provide in our test system. We have previously observed that silencing of mRNAs can be induced by delivery of homologous dsRNAs in tobacco protoplasts (data not shown). Importantly, this silencing was only

observed after several rounds of cell division. This could indicate that passage of nuclei through mitosis is required to enable functional silencing mediated by cytoplasmic inducers. The observation that TRV (tobacco rattle virus), which is able to infect growing points, is a much more efficient silencing inducer than PVX, which is largely absent from growing points (27), strongly supports the existence of a nuclear initiation process for mRNA silencing.

In contrast to our observations in non-silenced cells, we did not detect any virus-derived small RNAs upon introduction of chimeric viral RNAs with homology to silencing-target sequences in pre-silenced cells. Previous experiments showed that, while being efficient silencing targets, a range of chimeric viral RNAs, including STNV-L, accumulate to at least 100-fold higher levels as compared to the silenced *gnl* mRNA (9,22), for which siRNAs are readily detectable. Thus, if the chimeric viral RNA would be a substrate for appreciable amounts of siRNA production in pre-silenced cells we should have detected this in our experiments. Therefore, our findings indicate that, in pre-silenced cells, Dicer is not directly responsible for the elimination of the majority of incoming chimeric viral RNAs containing sequences of a pre-silenced mRNA. We conclude that silencing of such viral RNAs in pre-silenced cells predominantly proceeds via pre-existing, siRNA-programmed RISC complexes.

It is not clear which factors determine the relative contribution of Dicer and RISC to degradation of silencing targets. In the case presented here we observed that chimeric viral RNA clearly is an effective Dicer target upon infection of non-silenced cells, whereas it does not appear to be a direct Dicer target in pre-silenced cells. Lack of substantial direct Dicer activity on viral dsRNAs in protoplasts of silenced plants could be the result of the viral dsRNA level being too low to induce Dicer-like activity on these templates. Alternatively, viral RNAs being no substrate for both Dicer-dependent pathways could be a consequence of the Dicer enzyme being mainly sequestered to mRNA templates to ensure efficient mRNA silencing. A third explanation for our results could be that in pre-silenced cells Dicer is competent to process viral RNAs but cannot compete with pre-assembled RISC complexes. All these scenarios favour RISC as the preferred and/or quicker pathway for degradation of viral RNAs in pre-silenced cells.

It has been suggested that RdRP/Dicer and RISC are differentially deployed in function of the nature of the RNA template. For example, to explain why in plants transitivity is observed for transgenes but not for endogenous genes, Tang *et al.* (28) proposed that exogenous silencing triggers such as transgenes might be degraded via the RdRP/Dicer pathway, whereas endogenous targets would be degraded via RISC. However, our observation that secondary, transitive siRNAs corresponding to both a transgene and a co-suppressed endogenous gene accumulate in silenced cells (9) indicates that in silenced T17 plants the RdRP/Dicer pathway is active on both type of templates. This implies that deployment of silencing pathways does not solely depend on the nature of the template.

Our proposition that, upon introduction in pre-silenced cells, chimeric viral RNAs are predominantly degraded by RISC activity, guided by pre-existing mRNA derived siRNAs is in line with a mathematical model for RNA silencing

proposed by Bergstrom *et al.* (29). According to this model, siRNAs derived from primary targets are amplified during early stages of silencing to levels 'many-fold higher than their original prevalence'. In our model, this could create a pool of siRNAs that facilitates suppressed accumulation incoming chimeric viral RNAs without appreciable production of siRNAs from the incoming viral RNAs.

The work presented here demonstrates that the silencing machinery is being deployed in a flexible manner at multiple levels: both with respect to target selection within a certain sequence and with respect to deployment of a targeting route in function of silencing conditions. The mechanisms that regulate the selection and activity of the different RNA degradation pathways are not understood and make an interesting field for further investigation.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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