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Analysis of RNA silencing in agroinfiltrated leaves of *Nicotiana benthamiana* and *Nicotiana tabacum*

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Abstract

In this study we analyse several aspects of cytoplasmic RNA silencing by agroinfiltration of DNA constructs encoding single- and double-stranded RNAs derived from a *GFP* transgene and from the endogenous *Virp1* gene. Both types of inductors resulted after 2–4 days in much higher concentration of siRNAs in the agroinfiltrated zone than normally seen during systemic silencing. More specifically, infiltration of two transgene hairpin constructs resulted in elevated levels of siRNAs. However, differences between the two constructs were observed: the antisense–sense arrangement was more effective than the sense–antisense order. For both double-stranded forms, we observed a relative increase of the 24-mer size class of siRNAs. When a comparable hairpin construct of the endogenous *Virp1* gene was assayed, the portion of the 24-mer siRNA class remained low as observed for all kinds of single-stranded inducers. The lack of increase of *Virp1*-derived 24-mers was independent of the expression level, as demonstrated by agroinfiltration into a transgenic plant that overexpressed *Virp1* and showed the same pattern. Using transducer constructs, we could detect within a week transitive silencing from *GFP* to *GUS* sequences in the infiltrated zone and in either direction 5′–3′ and 3′–5′. Conversely, for the endogenous *Virp1* gene neither transitive silencing nor the induction of systemic silencing could be observed. These results are discussed in view of the current models of RNA silencing.

Introduction

RNA silencing is a complex process mediated by various classes of short RNAs that regulate gene expression in a sequence-specific manner. While microRNAs (miRNA) typically impair translation, short interfering RNA (siRNA) result in sequence-specific cleavage of cytoplasmic RNA or cause chromatin condensing, especially if expressed from repeat associated sequences, also called rasiRNA (repeat associated siRNA). Various aspects of RNA silencing have been reviewed recently (Ambros, 2004; Lippman and Martienssen, 2004; Meister and Tuschl, 2004; Mello and Conte, 2004; Novina and Sharp, 2004) including the special characteristics of this process in plants (Baulcombe, 2004). Here, the cytoplasmic non-cell-autonomous silencing pathway results in

breakdown of specific RNA sequences and provides a powerful protection system against invading plant RNA viruses.

Double-stranded RNA (ds RNA) is a key trigger for inducing sequence-specific cytoplasmic RNA degradation; the duplex form gets processed by a Dicer enzyme into siRNAs, which are then incorporated into the RNA-induced silencing complex (RISC). After ATP-dependent unwinding of the siRNA one of the two siRNA strands is removed and the complex acts as a sequence-specific ribonuclease that cleaves a single-stranded RNA target in a catalytic manner.

RNA silencing is an ancient biochemical process found in some unicellular organisms up to higher eukaryots. However, the mechanism shows considerable variations between kingdoms, plants especially seem to have developed a distinct, rather complex system of RNA silencing. Here, at least two forms of RNA-dependent RNA polymerase (RDR1 and RDR6), also known as RNA-directed RNA polymerases (RdRP), contribute to the different forms of RNA silencing, together with two size classes of siRNAs (consisting of 21/22 and 24 nucleotides) (Hamilton *et al*., 2002) that are derived from four types of Dicer (DCL1–4) enzymes (*C. elegans* and mammals have only one Dicer and *Drosophila melanogaster* two) (Schauer *et al*., 2002). DCL1 is a nuclear protein and processes miRNA precursors (Finnegan *et al*., 2003; Papp *et al*., 2003; Xie *et al*., 2004). DCL2 converts virus-derived (and other cytoplasmic) ds RNAs into siRNAs of the small size class, while DCL3 produces endogenously encoded siRNAs, which are primarily of the 24 nts class and which are required for chromatin condensing (DCL3 products are rasiRNAs) (Xie *et al*., 2004). The function of DCL4 is not clear.

RDRs are responsible for the synthesis of double-stranded RNA from single-stranded RNA. The template can be an aberrant RNA derived from a single copy gene, for example a defective mRNA lacking the poly(A)tail (unprimed synthesis) (Baulcombe, 2004), but also siRNA molecules may be used as primers to convert a ss RNA template into a ds RNA (Sijen *et al*., 2001; Tang *et al*., 2003), which is a substrate for Dicer cleavage. In such a way, secondary ds RNA may be generated and diced to secondary siRNAs, which can result in subsequent suppression of adjacent sequences. Such a spreading of silencing 'along a gene' has been described as transitive RNAi and has been investigated in several organisms (Sijen *et al*., 2001; Braunstein *et al*., 2002; Klahre *et al*., 2002; Vaistij *et al*., 2002; Alder *et al*., 2003; Chi *et al*., 2003; Roignant *et al*., 2003; Van Houdt *et al*., 2003; Vanitharani *et al*., 2003; Garcia-Perez *et al*., 2004; Vogt *et al*., 2004). Depending on the species studied, there are differences in how transitive RNAi is transmitted. Primer-dependent synthesis by RDR could easily explain transitive RNAi from the 3′ to the 5′ end of mRNA observed in some animals, but in plants additional spreading in the opposite direction has been noticed as well (Braunstein *et al*., 2002; Vaistij *et al*., 2002). Another interesting characteristic of transitive RNAi is the different behaviour depending on the nature of genes involved. Transgene sequences show spreading of silencing, while endogens seem to be protected. The absence of transitivity was demonstrated when genes encoding phytoene desaturase (PDS) and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) were investigated (Vaistij *et al*., 2002), while limited transitivity was observed for two β-glucuronidase (*GUS*) transgene constructs (English *et al*., 1996; Elmayan *et al*., 1998; Wang *et al*., 2001). It was proposed that certain structural features of the genes or association with specific proteins may inhibit RDR (Vaistij *et al*., 2002), the problem however, remains unsolved, because in other studies the tobacco β-1,3-glucanase gene served as a template for the production of secondary siRNAs (Sanders *et al*., 2002).

After initiation and amplification of the cytoplasmic RNA silencing pathway, spreading to neighbouring cells and systemically throughout the plant is the final step of the process (Palauqui *et al*., 1997; Voinnet and Baulcombe, 1997; Voinnet *et al*., 1998). Short-distance spread of silencing is limited to 10–15 cells and most likely mediated by the 21/22-mer class of siRNAs without the need of homologous transcripts (Himber *et al*., 2003). By contrast, long-distance transmission of the silencing signal requires an amplification step, involving an RDR.

In plants, cytoplasmic RNA silencing can be induced efficiently by agroinfiltration, a strategy for transient expression of T-DNA vectors after delivery by *Agrobacterium tumefaciens*. The transiently expressed DNA encodes either a single- or double-stranded RNA, which is typically a hairpin (hp) RNA. Because they provide a rapid, versatile and convenient way for achieving a very high level

of gene expression in a distinct and defined zone of a leaf, *Agrobacterium*-mediated transient expression systems have been useful for inducing silencing processes (Voinnet and Baulcombe, 1997; Johansen and Carrington, 2001) and for dissecting the mechanism of gene silencing, especially concerning its suppressors, systemic silencing signal, but also for simple protein purification (Johansen and Carrington, 2001; Mlotshwa *et al*., 2002; Tenllado *et al*., 2003; Voinnet *et al*., 2003).

In this report we describe the onset of cytoplasmic RNA silencing with various single- and double-stranded RNA inducers derived from transgenes and an endogenous gene in different transgenic and non-transgenic *Nicotiana* species. We analyse in a systematic manner the nature of siRNAs generated and their time course of appearance in agroinfiltrated tissues. In addition we characterise the potential of transitive silencing within the agroinfiltrated zone.

Materials and methods

Vector construction

The hybrid (*GUS*:*GFP*, *GFP*:*GUS*) and hairpin *GFP* constructs (Figure 2) are based on plasmid pFGC5941 that was kindly donated by the Jorgensen Lab (Napoli, C. and Atkinson, R. ChromDB http://ag.arizona.edu/chromatin/fgc5941.html).

Two hybrid constructs (with *GUS*:*GFP* order) were prepared by cloning separate PCR products with restriction sites added by appropriate primers into the polylinker. A *GUS* cDNA fragment of about 660 bp (PCR-amplified with primers 5′-CGA CTA GTG GCG CTG GAG CAT CAG GGC GGC TAT A and 5′-GCG GAT CCA TTT AAA TGG TAC GGT AGG AGT TGG CCC CT) was digested and ligated to a *Asc*I–*Bam*HI-cleaved pFGC5941 vector replacing the ChSA intron. Next, a 630-bp *GFP* cDNA fragment was amplified in a similar way (5′-ATA CTA GTG GCG CGC CCA CTG GAG TTG TCC CAA TTC and 5′-GCG GAT CCA TTT AAA TAC TCA AGA AGG ACC ATG TGG), digested and ligated to *Bam*HI–*Spe*I sites close to the *GUS* fragment. In addition, the *GFP* fragment was amplified using the similar DNA primers, but containing *Bam*HI and *Swa*I restriction sites in the upper primer and *Spe*I, *Asc*I sites in the lower one (ATG GAT CCA TTT AAA TCA CTG GAG TTG TCC CAA TTC and GCA CTA GTG GCG CGC CAC TCA AGA AGG ACC ATG TGG, respectively). This permitted cloning of the *GFP* fragment in the opposite orientation.

In order to make the *GFP*:*GUS* constructs, the entire recombinant cDNA hybrid (*GUS*:*GFP*) sequence was PCR-amplified using the appropriate flanking primers; the PCR product was cloned into pGEM T-easy vector (Promega). Then, *Eco*RI fragments were subcloned into pBluescript SK (Stratagene) vector and selected *Spe*I–*Xho*I fragments, having the hybrid in a correct orientation, were cloned back into pFGC5941 plasmid. Finally, four constructs were obtained, which differ in order and orientation of *GUS* and *GFP* fragments, as shown in Figure 2.

The two *GFP* hairpin constructs (pANe59I and pANe59R, called for simplification as hpGFP/As-S and hpGFP/S-As under Results) were prepared in a two-step cloning process in pFGC5941 plasmid. Prior to the construction of hairpins, the *Spe*I restriction site in the ChSA intron of pFGC5941 vector was removed. The PCR-amplified *GFP* cDNA fragments were first cleaved at the inner (*Asc*I and *Swa*I) and then the outer restriction sites (*Spe*I and *Bam*HI) and cloned on either side of the intron sequence. In order to make the hp*GFP* in s-as arrangement the upper primer with the *Spe*I and *Asc*I restriction sites and the lower primer with the *Bam*HI and *Swa*I sites were used. In order to make the hpGFP/As-S construct *Bam*HI and *Swa*I were included in the upper primer and *Spe*I and *Asc*I in the lower one.

Virp1 sense overexpressing construct: A 2203 bp *Rsa*I fragment containing the whole *Virp1* gene from clone 8 (Martinez de Alba *et al*., 2003) was introduced in the *Sma*I site of pART7 vector generating pART7/X1. Then the entire expression cassette of pART7/X1 containing the CaMV *35S* promoter, the *Virp1* gene sequence and the *ocs* terminator, was extracted from the plasmid by a *Not*I digest and subcloned in a *Not*I site of the binary vector pART27 (Gleave, 1992), producing transformation vector pART27/X1.

Hairpin producing construct: For the generation of the *Virp1* suppressor, the *Bam*HI 734 bp fragment containing the bromodomain sequence and the RNA-binding domain sequence of the gene was subcloned in sense and antisense orientation behind a CaMV 35S promoter of the

pART7 vector producing vector pART7/phX1. The two *Virp1* fragments of the inverted repeat were separated by a 597 bp intron sequence (from the *Glutamate-Dehydrogenase E.C. 1.4.1.2* gene of *Vitis vinifera*, kind donation of K. Roubelakis-Angelakis). The expression cassette of pART7/Virp1 including the *35S* promoter, the *Virp1* inverted repeat and the *ocs* terminator was subcloned in pART27 as described for pART7/X1.

Plant transformation

For our experiments we used *Nicotiana tabacum* (*N.tabacum*) Xanthi *GFP*, *Nicotiana benthamiana* (*N. benthamiana*) wild type, *N. benthamiana* GFP line 16c, *N. benthamiana Virp1*.

The *N. benthamiana GFP* line 16c was kindly donated by D. Baulcombe, the Sainsbury Laboratory, John Innes Centre, Norwich, UK. *N. tabacum* Xanthi transgenic for *GFP* was generated by *Agrobacterium*-mediated transformation. The pBIN 35S-mgfp5-ER construct (courtesy of J. Hasellof (MRC Cambridge)) was transferred into *Agrobacterium tumefaciens* strain LBA4404 *via* triparental mating with *E. coli* strain HB101 containing pRK2013 (Bevan, 1984). Plants were transformed by the leaf disc transformation procedure (Horsh *et al.*, 1988). Shoots that rooted in the presence of 100 μg/ml kanamycin were considered to be transgenic. The proof of transformation was obtained by fluorescence measurement (excitation wavelength 475 nm, emission 510 nm).

Nicotiana benthamiana Virp1 plants were transformed with the pART27/X1 vector after introduction into *A. tumefaciens* strain LBA4404 by tri-parental mating, as described before (Kalantidis *et al.*, 2002). Explants and plants were grown at 25 and 23 °C – day, respectively, and 18 °C night in the growth chamber with a 16 h photoperiod provided by cool white fluorescent tube lights to give 90 μmol m^{-2} s^{-1} PAR. Plantlets were then transferred to the greenhouse where they were grown to maturity.

Agroinfiltration

All T-DNA constructs were introduced into *A. tumefaciens* LBA 4404 by electroporation as described previously (Nagel *et al.*, 1990). Agrobacteria were grown overnight in LB medium with the appropriate antibiotics and 20 μM acetosyringon; then they were briefly spun down and re-suspended in MMA medium (MS salts, 10 mM MES, pH 5.6, 200 μM acetosyringon) and incubated for at least 1 h at 28 °C. Subsequently, the bacteria were washed twice with 10 mM $MgCl_2$ and re-suspended in $MgCl_2$ to a low OD_{600} of about 0.3–0.4. Injection of the diluted bacteria was done as described before (Schob *et al.*, 1997). After agroinfiltration the plants were kept in constant conditions, in the growth chamber at 23 °C – day and 18 °C – night with a 16 h photoperiod. For monitoring the expression or silencing of *GFP* a handheld 100 W long-wavelength UV lamp (B1000AP; Ultraviolet Products) was used, and a Nikon COOLPIX 990 digital camera was used for photography.

RNA extraction and Northern blot analysis

Total RNA was isolated with acidic phenol (pH 4.0) (Boutla *et al.*, 2002). Small RNA enrichment was achieved by re-suspension of the RNA pellet in 8 M LiCl according to previously published methods (Papaefthimiou *et al.*, 2001; Kalantidis *et al.*, 2002). Northern analyses of mRNA levels and of siRNA detection were also performed as described before (Papaefthimiou *et al.*, 2001; Kalantidis *et al.*, 2002). For probing, cDNA fragments were labelled by random primed incorporation of [^{32}-P]dATP and dCTP (RadPrime DNA Labelling System, Invitrogen). The control U6 and U1 snRNA antisense probes were obtained as described previously (Denti *et al.*, 2004), U6 by transcribing the *Eco*RI-linearised plasmid containing the mouse U6 snRNA gene with T7 polymerase and U1 antisense probe (potato) by transcribing the *Eco*RI-linearised plasmid pU1EH with SP6 RNA polymerase (Vaux *et al.*, 1992). DNA oligonucleotide of antisense microRNA sequence (*At* miR167, sequence TAGATCATGCTGGCAGCTTCA) labelled with [γ^{32}-P]ATP in PNK reaction was used as loading standard of siRNAs in Figure 3A.

Results

As a first step we compared the amounts of siRNAs that can be obtained by induction of cytoplasmic RNA silencing via agroinfiltration

with the concentration detectable in a systemically silenced plant. We agroinfiltrated in *N. benthamiana* transgenic for *GFP* (16c) two different DNA constructs that either expressed a ss *GFP* RNA or a ds RNA hairpin construct. Four days after the agroinfiltration we analysed the infiltrated zone for siRNAs. In parallel, we extracted RNAs from a 16c plant that had been systemically silenced by the infiltration of a hairpin construct about three weeks before the experiments. Figure 1A shows that the highest concentration of siRNAs was detectable in leaves overexpressing the hairpin construct; it also shows that the concentration of siRNAs induced by the ss RNA is still dramatically higher compared with the concentration found in a systemically silenced leaf. Systemic silencing was detected visually by loss of fluorescence initially along the veins which at this stage corresponded with reduced *GFP* mRNA levels (Figure 1B). Later silencing spread throughout the leaf and at this stage *GFP* mRNA could not be detected anymore (not shown).

Temporal analysis of the siRNA formation after agroinfiltration of ss and ds RNA transgene constructs

For a more detailed analysis of what size classes of siRNAs are generated after agroinfiltration and at what times, we used three sorts of inducer constructs: the plasmid vector that expressed ss *GFP* full size sense RNA and two different *GFP* hairpin constructs, which resulted in the expression of ds *GFP* RNA of about 630 bp covering a large portion of the *GFP* gene sequence (Figure 2A, C). However, the two latter constructs differed in the

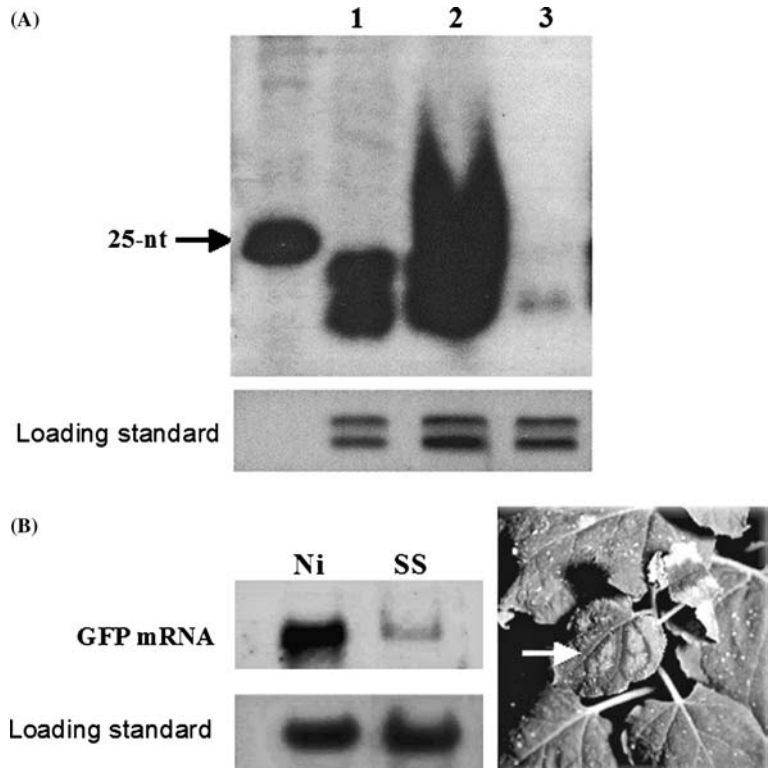

Figure 1. (A) Accumulation of *GFP*-specific siRNAs in *N. benthamiana* line 16c after different initiation of cytoplasmic RNA silencing. For Northern blot analysis comparable amounts of RNA extracts were loaded, which were derived from zones that had been agroinfiltrated with a DNA construct expressing ss *GFP* (lane 1), a hp *GFP* construct (lane 2) or from a systemically silenced plant (lane 3). The marker lane M contains an unrelated 25-mer RNA marker. Hybridisation to U1 RNA provides a loading control. (B) *GFP* mRNA levels in *N. benthamiana* line 16c. Non-infiltrated and systemically silenced leaves are marked as Ni and SS, respectively. Hybridisation to 18S RNA is a loading standard. The phenotype of the systemically silenced plant, seen in UV light, is presented aside. The arrow indicates initiation of the silencing process in a new leaf.

order of the sense and antisense strand. In plasmid pANe59I (hereinafter called hpGFP/As-S for simplification) the CaMV *35S* promoter drives the expression of an antisense–sense construct, while in plasmid pANe59R (hpGFP/S-As) the order is inversed (Figure 2A). We used *N. benthamiana* 16c plants, which had about eight fully developed leaves, and the three constructs were each agroinfiltrated into the largest four leaves of individual plants. To eliminate environmental influences the plants were kept in growth chambers. After 2, 4, 6 and 8 days one leaf from each plant was extracted and analysed for the generation of siRNAs in the infiltrated zone. The Northern blot analysis of one infiltration experiment is summarised in Figure 3. The earliest detection of siRNAs after the infiltration of the construct that expressed single-stranded *GFP* RNA was slightly variable. The experiment was repeated several times and in some experiments no detectable levels of siRNAs could be observed at day 2 (Figure 3A), while, in other assays this was possible (not shown). This suggests that generation of detectable amounts of siRNAs occurred at day 2 or slightly thereafter. The concentration of siRNAs peaked at day four and stabilised at a lower level at days six and eight (Figure 3A, left). At all four times analysed, we detected primarily siRNAs of the 21/22-mer class and only low amounts of the 24-mer siRNAs. Although the above pattern of siRNAs is the usual for ss constructs it has to be noticed that exceptionally, in relevant experiments, a relatively high amount of the 24nt class has also been detected (not shown). As expected, the infiltration of the ds constructs (Figure 3A, middle and left) resulted in a much higher concentration of siRNAs, because ds RNA is a direct substrate for the Dicer enzymes. Besides the increase of the 21/22-mer class of siRNAs, we saw a striking increase of the 24-mers, which reached levels similar to or even higher than the 21- or 22-mers. In addition, we noticed that the hpGFP/As-S was a stronger inducer for the generation of siRNAs than the comparable hpGFP/S-As construct, both expressed from a *35S* promoter. Not only was the concentration of siRNAs generally higher for construct hpGFP/As-S, but siRNAs were also generated earlier, so that already at day two high quantities of siRNAs were detectable. We could confirm these findings in two further and independent experiments. In addition, we infiltrated the two hairpin constructs that differ in the order of sense and antisense sequences into 16c plants of different ages and we found that the onset of siRNAs formation took longer in older plants. However, in all cases the hpGFP/As-S construct resulted in a higher concentration of siRNAs at earlier times (data not shown). In accordance to *GFP*-specific siRNAs detected following agroinfil-

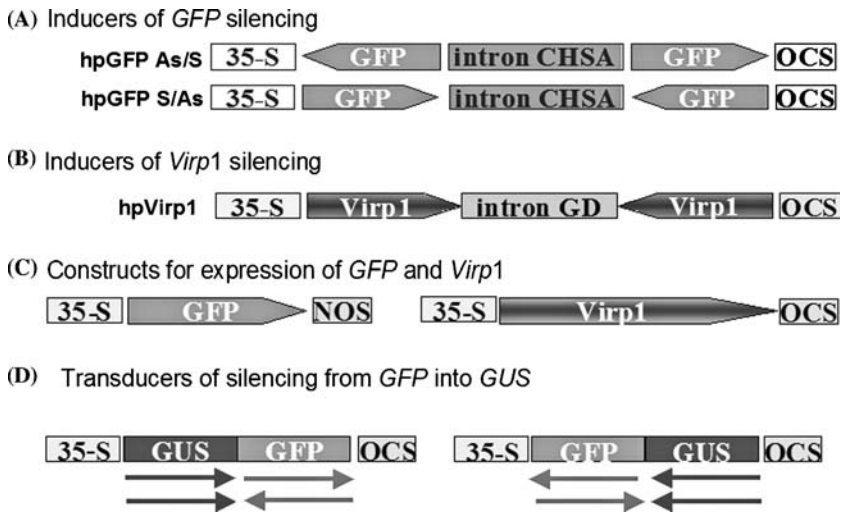

Figure 2. Schematic maps of DNA constructs designed for the induction of *GFP* silencing (A), *Virp1* silencing (B), expression of *GFP* and *Virp1* genes (C) and transduction of the degradation process from *GFP* into *GUS* sequences (D). Sense- or antisense orientation of the *GFP* and *GUS* fragments are indicated by arrows. Positions of the CaMV *35S* promoter (35-S), the OCS or NOS terminators and the chalcone synthase (ChSA) or glutamate-dehydrogenase (GD) introns are indicated as well.

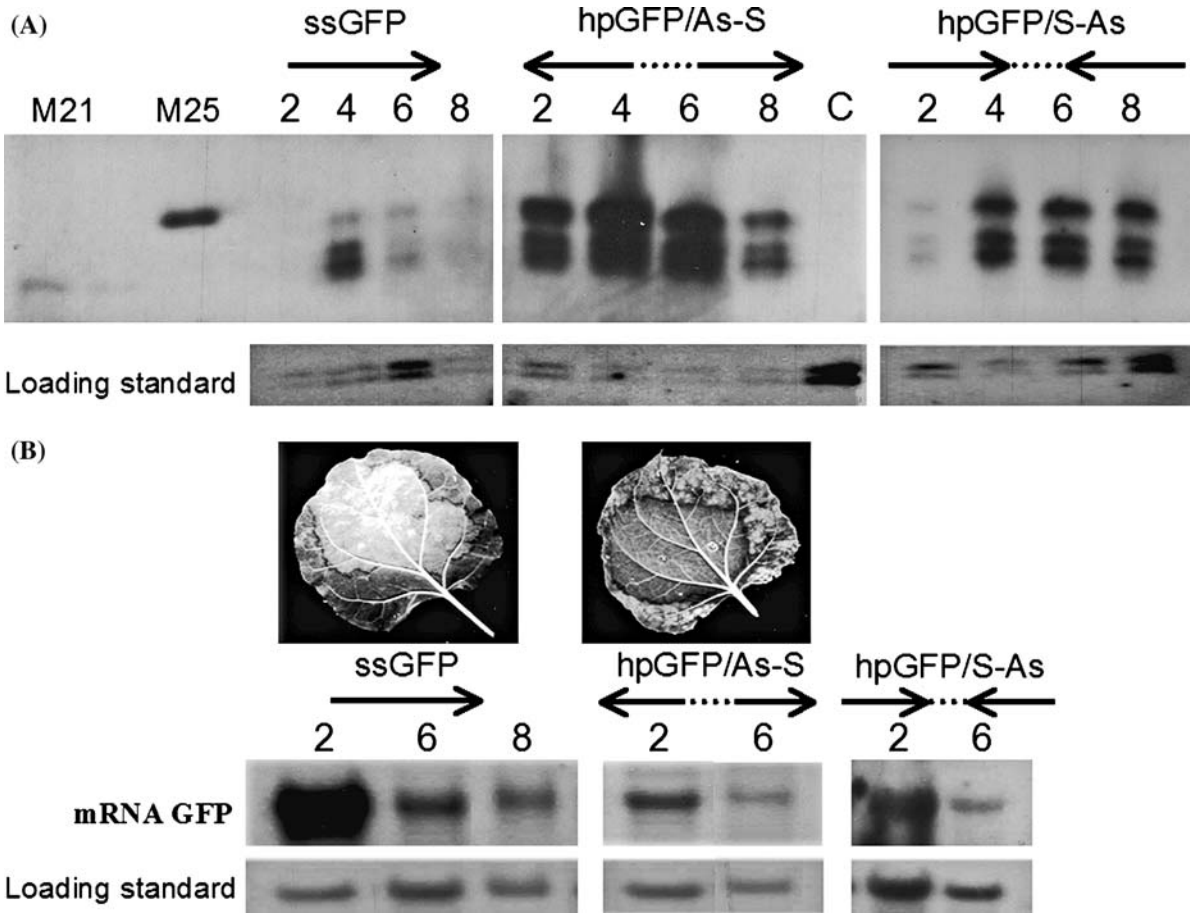

Figure 3. (A) Time course analysis of siRNA generation after agroinfiltration of different *GFP* constructs into *N. benthamiana* line 16c. The constructs for infiltration are indicated; the numbers above the lanes indicate the day of analysis after infiltration; lanes M21 and M25 correspond to marker RNAs of 21 and 25 bases; (C) is an RNA extract from a non-infiltrated control. Hybridisation to *At* miR 167 provides a loading control. (B) *GFP* mRNA levels at certain time points after agroinfiltration of different *GFP* constructs into *N. benthamiana* line 16c. The constructs are indicated as in (A). Hybridisation to 18S RNA was used as a loading control. Images above blots present the leaf phenotypes. The *GFP* silencing phenotype produced by the GFP/S-As hairpin construct is identical to the one produced by the GFP/As-S construct.

tration with the above constructs *GFP* mRNA levels were found to gradually decrease (Figure 3B).

In order to test whether the pattern of siRNA formation could be reproduced in a different species, we repeated the infiltration experiment with a *GFP* transgenic line of *N. tabacum*. Agroinfiltration of the same three *GFP* constructs as above confirmed the results obtained with line 16c although quantitative differences in the timing of siRNA generation can be observed between the two species. Once again the hpGFP/As-S construct was a much better inducer for the formation of siRNAs than hpGFP/S-As (Figure 4). It should be noted that also the pattern of the relative concentration of 24-mer siRNAs versus 21- and 22-mer could be reproduced in *N. tabacum*. In order to address whether the presence of transgene transcribed ss sense *GFP* RNA plays a role in the different efficiency of the two constructs to generate secondary siRNAs we co-infiltrated the two hp constructs into wt *N. benthamiana*. However, again infiltration of the hpGFP/As-S construct gave higher amounts of siRNAs in wt plants (not shown).

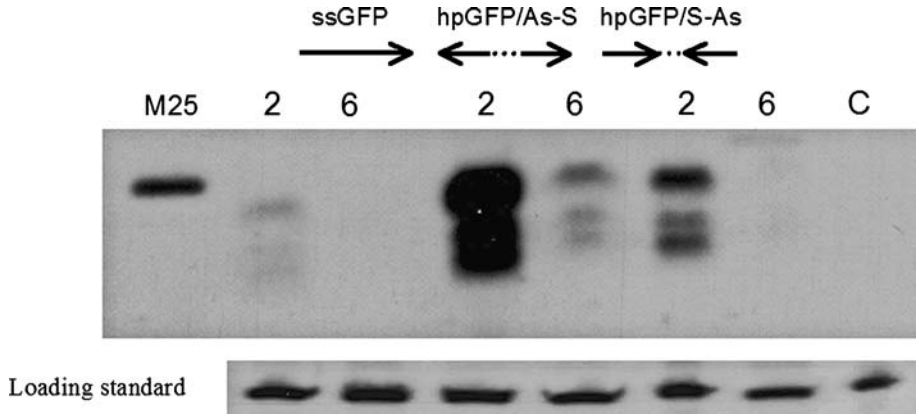

Figure 4. Time course analysis of siRNA generation after agroinfiltration of different *GFP* constructs into *N. tabacum* Xanthi. The constructs for infiltration are indicated; the numbers above the lanes indicate the day of analysis after infiltration; lane M25 corresponds to a 25-mer marker RNA; C is an RNA extract from a non-infiltrated control. Hybridisation to U6 RNA provides a loading control.

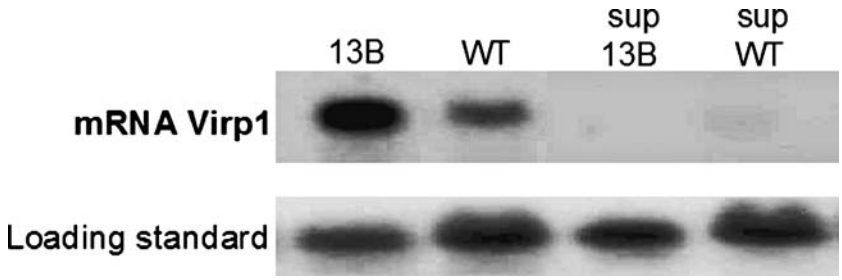

Figure 5. The *Virp1* mRNA levels in *N. benthamiana Virp1* overexpressing (13B) and wilde type (WT) plants before and after suppression with a hairpin construct (sup 13B and sup WT, respectively). Hybridisation to U6 RNA provides a loading control.

Agroinfiltration of endogenous ss and ds RNAs

As a next step we repeated the analysis of the formation of siRNAs after agroinfiltration of ss and ds constructs for an endogenous gene rather than a transgene. For that purpose we used sequences encoding the viroid binding protein (*Virp1*) (Martinez de Alba *et al*., 2003), which has an equivalent gene in *N. benthamiana* with a great degree of sequence similarity (Kalantidis *et al*; unpublished). Compared to the *GFP* transgene, the endogenous *Virp1* gene, which is most likely a transcription factor, is expressed at much lower levels (Figure 5, lane 2). We infiltrated a ss sense and a ds hp*Virp1* construct of the s-as arrangement into *N. benthamiana*. Figure 6A shows that the infiltrated ss *Virp1* RNA was also able to induce the formation of siRNAs. In accordance with the lower level of *Virp1* expression (compared to *GFP* in 16c) the concentration of siRNAs was lower. As for ss *GFP* RNA mostly 21/22-mer siRNAs could be detected, but only traces of 24-mers. The infiltration of a ds *Virp1* construct again resulted in a higher concentration of siRNAs. However, as for the ss *Virp1* RNA we saw mainly the 21/22-mer class of siRNAs and only a low concentration of 24-mers, which is at variance to the outcome of the analysis of ds *GFP* sequences. In order to find out whether siRNA formation was influenced by expression levels of the target *Virp1* gene we repeated the agroinfiltration of the *Virp1* constructs with a transgenic *N. benthamiana* line that overexpresses *Virp1* with a high steady state level of its mRNA (Figure 5, lane 1). Figure 6C shows the same pattern of siRNAs as in the wt background (the difference in timing is due to the older age of the plants used for this experiment); no increase in the 24-mer siRNAs was observed with the hp construct, in contrast to the *GFP* plants. These data

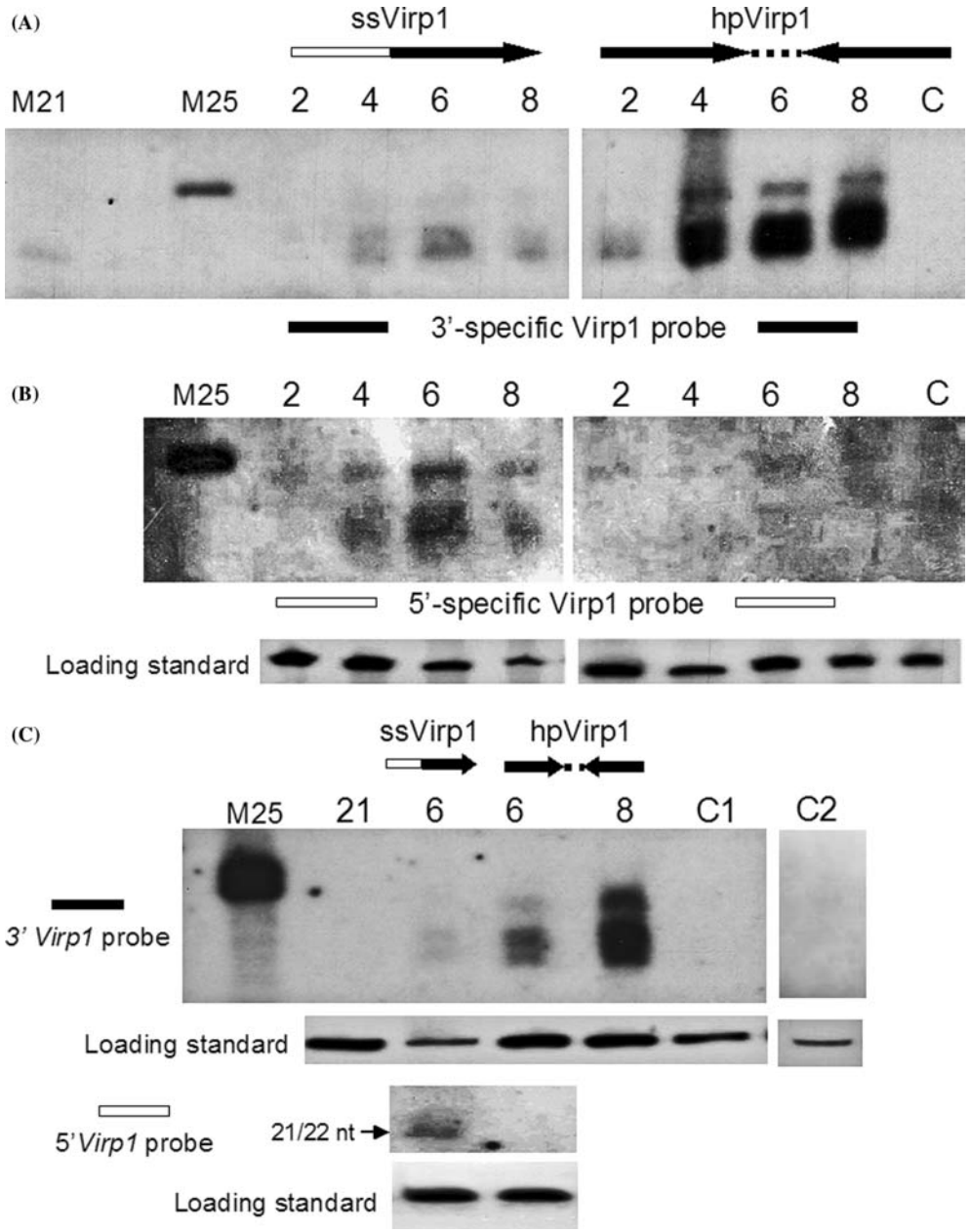

Figure 6. Time course analysis of siRNA generation after agroinfiltration of two different *Virp1* constructs into *N. benthamiana* wt (A, B) and *Virp1* overexpressing plant (C). The maps of the constructs are given schematically; the ss *Virp1* construct comprises the entire gene, while the hp construct contains the central and 3′-terminal part. Hybridisation was done with either a 3′ terminal (A) or a 5′ terminal probe (B). Numbers above the lanes indicate the day of analysis after infiltration; lane M25 correspond to a 25-mer marker RNA and C is an RNA extract from a non-infiltrated control. Hybridisation to U6 RNA provides a loading control. (C) Upper panel: lane 21 is an analysis of upper (systemic) leaves 21 days after the agroinfiltration of the hp construct – no siRNAs are detectable there. C1 is an RNA extract from a non-infiltrated control negative control, C2 is an RNA extract from a *Virp1* overexpressing plant agroinfiltrated with an empty vector and hybridised with 3′ *Virp1* probe. Lower panel: samples from day 6 p.i. with *Virp*-sense and *Virp1*-hairpin, respectively, were hybridised with 5′ terminal probe to detect secondary siRNAs. *Virp1*-specific siRNAs are detected only in the case of ss *Virp1* construct. Hybridisation to U6 RNA provides a loading control. The position of 21/22-nt siRNA is indicated by an arrow.

suggest that the nature of the gene rather than its relative expression level determines the pattern of siRNAs. In both wt and *Virp1* overexpressing plants a sharp decrease of *Virp1* mRNA levels could be detected 6 days p.i. of the hairpin construct (Figure 5). In addition, we took advantage of the fact that the ds *Virp1* construct used for agroinfiltration comprised only the downstream part of the gene, so that we could test for the spreading of silencing into the upstream part in the presence of massive amounts of siRNAs. When we used a probe specific for the upstream part of the gene we could not detect *Virp1*-derived siRNAs in wt plant, suggesting that the ds RNA was in this case not able to induce transitive RNAi. After long exposure (two weeks) a faint signal at the 24 nt size class could be observed for two of the samples (Figure 6B, day 2 and 6 p.i.) However, it is not possible to conclude whether this is due to background hybridisation or to true *Virp1* signal.

Next we intended to investigate whether the lack of transitive silencing for the endogenous gene is due to the 'endogenous' gene sequence *per se*, having some intrinsic characteristics that protect it from transitive silencing, or whether this 'insulation' is rather the result of the native context of the gene. In order to address this issue we repeated the above agroinfiltartions in a transgenic *N. benthamiana* line that in addition to the endogenous *Virp1* genes carries also an additional *Virp1* copy driven by a *35S* promoter (Kalantidis *et al*. unpublished results). Using a 3′ *Virp1* specific probe which detects both primary and secondary siRNAs, we found little difference in the quality or quantity of siRNAs detected between wt (see above, Figure 6A) and overexpressing plants (Figure 6C upper panel). However, similar to the result obtained in wt background, hybridisation with a 5′ specific probe that detects only secondary siRNAs did not reveal siRNAs in leaves of the transgenic plant overexpressing *Virp1* that had been agroinfiltrated with ss *Virp1* and hp*Virp1* constructs (Figure 6C lower panel, compare with Figure 6B). This finding suggests that there might be a certain 'endogenous feature' of the sequence that protects it from transitive silencing. Consistent with this, in neither of the two plants could we detect any *Virp1*-specific siRNAs in the top leaves that were harvested three weeks after the agroinfiltration with either ds (Figure 6C, lane 2) or ss RNA (not shown) constructs. This means that silencing of the endogenous sequence was restricted to the agroinfiltrated area and did not extend neither along a gene nor throughout the plant.

Transitive RNAi of exogenous gene sequences in agroinfiltrated leaf tissue

Since endogenous genes and transgenes behave differently in transitive RNAi (English *et al*., 1996; Elmayan *et al*., 1998; Wang *et al*., 2001; Sanders *et al*., 2002; Vaistij *et al*., 2002) we intended to see whether these results could be recapitulated by agroinfiltration experiments, taking advantage of the high concentration of siRNAs obtainable from the hairpin constructs. For this purpose we used four different transducers that express ss *GFP* sequences of either sense or antisense polarity fused to ss *GUS* sequences (Figure 2D). Two of the four constructs contained the *GFP* sequences at the 5′ end of the recombinant RNA, while the other two contained them at their 3′ end. Since siRNAs can be induced also in wt plants after infiltration of ss RNA producing constructs (Szittya *et al*., 2003; Bucher *et al*., 2004) we determined first whether in our test system and with our lower concentrations of infiltrated agrobacteria we would detect any siRNAs after infiltrating any of the four transducers separately in *N. benthamiana* wt plants. For none of the four constructs could we detect any *GFP*- or *GUS*-specific siRNAs in the infiltrated zone (data not shown). Next, we infiltrated each of these transducers individually in the same concentration, however, together with the hpGFP/As-S hairpin construct, which served as a donor of *GFP*-specific siRNAs. One week after agroinfiltration, we could detect in the infiltrated zone *GUS*-specific siRNAs (Figure 7A). We could not observe any difference in concentration or pattern of *GUS*-specific siRNAs depending on the arrangement or orientation of *GUS* and *GFP* sequences. As a control we stripped the membrane and re-hybridised it with the *GFP*-specific probe. This confirmed the presence of high quantities of the *GFP*-specific siRNAs. As an additional control we repeated the stripping of the membrane, which was re-probed with the U1-specific probe to confirm equal loading of the RNA extracts. Collectively these data show that transitive silencing can be induced in the agroinfiltrated zone by siRNAs specific for sequences of the transducer

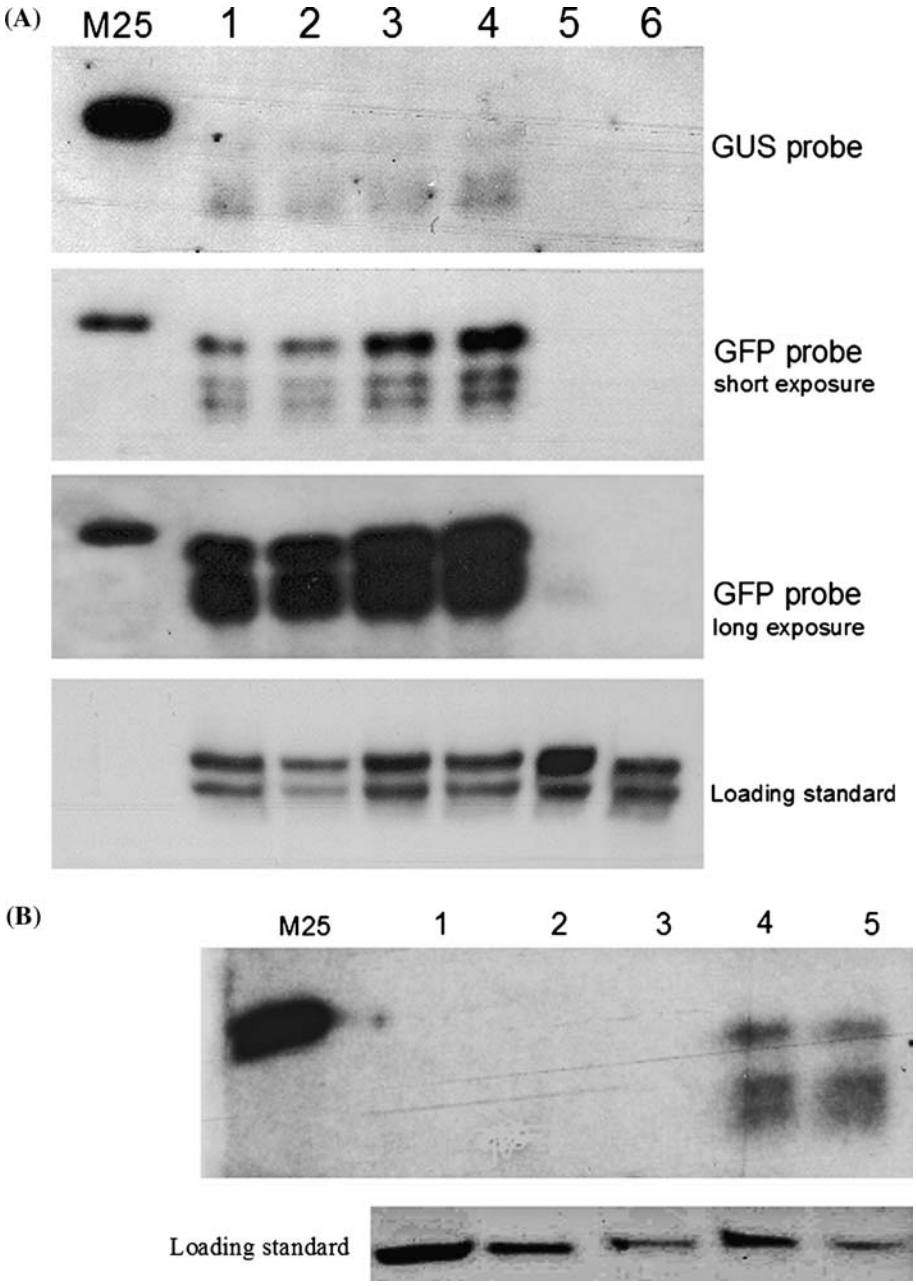

Figure 7. (A) Analysis of transitive silencing within the agroinfiltrated zone of *N. benthamiana* line 16c. Lanes 1–4: leaves agroinfiltrated the hpGFP construct together the sense*GUS*–antisense*GFP* (1), sense*GFP*–antisense*GUS* (2), sense*GUS*–sense*GFP* (3), antisense*GFP*–antisense*GUS* (4) construct (compare Figure 2); lane 5 extracts form a systemically silenced *GFP* leaf; lane 6 from non-infiltrated 16C control plant. The same blot was first hybridised to detect *GUS*-specific siRNAs, which are secondary siRNA, that are detectable in lanes 1–4, but not in 5 and 6. After stripping, the membrane was hybridised with a *GFP*-specific probe to visualise the inducing *GFP* siRNAs. Hybridisations to U1 RNA provide a loading control. (B) As a control, different constructs were agroinfiltrated into appropriate plants as follows: 1 – *GFP* full size sense construct in wt plant; 2 – hybrid *GFP:GUS* in wt plant; 3 – hybrid *GUS:GFP* in wt plant; 4 and 5 positive controls, 4 – hybrid *GFP:GUS* + hp*GFP* in *GFP* plant; 5 – hybrid *GUS:GFP* + hp*GFP* in *GFP* plant; RNA was extracted from the agroinfiltrated areas 6 days p.i. and was hybridised to *GFP* DNA probe. Hybridisation with U6 RNA is used as loading control.

constructs, independently of their location and orientation.

A striking observation for the transitive silencing with transgenes was, however, that the pattern of the *GUS* siRNAs differed from that of the *GFP* siRNAs. For *GUS* almost only 21-mers and 22-mers were detectable, but only traces of 24-mers, while the concentration of *GFP* 24-mers was as high as in the previous infiltration experiments with ds *GFP* constructs. The pattern observed for *GUS* siRNAs, which are the result of transitive RNA silencing, is comparable to the pattern we detected when constructs expressing ss RNA were infiltrated (Figure 3A, 6A). Thus, not only the nature of the gene, but also the total amount of siRNAs, most likely reflecting the amounts of ds RNA produced, influenced the relative proportion of the two size classes 21/22 and 24 nucleotides. A high concentration of siRNAs was seen only for transgenes and only if hairpin constructs were agroinfiltrated. Agroinfiltration of sense *GFP* and hybrid (*GFP-GUS*, *GUS-GFP*) constructs into wt leaves did not produce any detectable siRNAs (Figure 7B).

Discussion

Agroinfiltration is a powerful method to study processes connected with RNA silencing. In most cases agroinfiltration is used to initiate systemic silencing or to monitor the effect of suppressor genes. The infiltration of hairpin constructs is especially effective, because their ds RNA can be processed directly to siRNAs, but constructs expressing ss RNA can also be used to induce silencing (Voinnet and Baulcombe, 1997; Johansen and Carrington, 2001; Mlotshwa *et al*., 2002; Tenllado *et al*., 2003; Voinnet *et al*., 2003). So far, however, only few data are available on the timing of siRNA generation in the agroinfiltrated zone (Johansen and Carrington, 2001), and no analysis of how siRNAs derived from agroinfiltration compare in quantity and quality with those detected during conventional forms of systemic silencing.

Timing and pattern of siRNAs generated after agroinfiltration

We found that the production of siRNAs starts as early as 2 days after agroinfiltration, with a peak concentration at day 4. It is remarkable that also ss RNA in addition to ds RNA can also induce high amounts of siRNAs at early time points, because that arrangement requires generation of ds RNA by an RDR. We could show that also during agroinfiltration, a non-plant gene (here *GFP*) is easier to silence than an endogenously expressed gene. Similar observations have been made for the induction of systemic silencing (Vaucheret *et al*., 1998; Sanders *et al*., 2002). The most striking observations were variations in the relative proportion of the short and long forms of siRNAs for these two classes of genes, depending on the nature of the gene and on whether ss or ds inducers had been used. Only when delivery of hp *GFP* RNA resulted in a very high concentration of siRNAs, did we also see a change in the siRNA pattern, with more of the 24-mer class of siRNA being generated than the 21/22-mers. However, comparable hp *Virp1* RNAs delivered always only relatively few 24-mer siRNAs, even in highly expressing *Virp1* plants. The higher proportion of the 24-mer class of siRNA for a transgene during agroinfiltration is consistent with the conclusion that this size class is involved in the transmission of silencing (Hamilton *et al*., 2002). *GFP* plants were very easily silenced systemically, whereas in case of endogenous *Virp1* (both in wt and *Virp1*-overexpressing plants) silencing did not spread outside the agroinfiltration area, which is in agreement with the low concentration of 24-mer siRNA in those plants. The appearance of the 24-mer class of RNA could be explained by possible saturation of the Dicer system that generates 21/22-mer siRNA(likely to be DCL2, (Xie *et al*., 2004). In this case the steady state concentration of ds RNA might be higher so that a second Dicer enzyme (DCL3, (Xie *et al*., 2004)), which works normally at low activity could be more noticeable (although sometimes hardly detectable, there is always some amount of 24-mers). The Dicer that gets active at high concentrations of ds RNA is likely to be DCL3, since it is considered responsible for the generation of longer (24 nt) (Xie *et al*., 2004). Besides their presumed role in chromatin condensing, the 24-mer siRNA could be therefore also a cellular indicator that Dicer processing of 21/22-mers is running at maximum rate.

Differences in ds RNA constructs

Apart from the fact that hp *GFP* constructs gave more 24-nt siRNAs, there was a difference depending on the orientation of the hairpin. The

antisense–sense orientation (hpGFP/As-S) were consistently more efficient than its equivalent in the sense–antisense orientation (hpGFP/S-As). In accordance to this, Etienne Bucher and Marcel Prins (manuscript in preparation) observed that hairpin constructs designed against four tospoviruses were much more efficient in the As-S orientation (81% transgenic resistant lines) than in the S-As orientation (63% transgenic resistant lines). If this is indeed a general phenomenon one explanation could be that the direction of Dicer processing might play a role, because one side is protected by the hairpin. When the As-S construct is diced, the first siRNAs that are generated originate from the 'downstream' region of the gene. Therefore, the antisense strand of the siRNA might be able to prime the synthesis of ds RNA by an RDR. In contrast, if the S-As sense hairpin is infiltrated, the first appearing siRNAs are derived from the upstream part of the target gene. Here they can induce only short pieces of ds RNA. As a result, more siRNAs could be generated by the As-S construct. However, this does not explain why also in wt plants (in the absence of a *GFP* transgene) the As-S construct resulted in more siRNAs and even when we co-infiltrated the hp with a ss antisense construct the outcome was identical, as this should reverse the efficiencies if the above explanation were valid. Therefore, one has to take in consideration that also different hp constructs vary in their efficacy to induce silencing. Factors such as variation in transcription efficiency, splicing of the stuffer intron, export to the cytoplasm, scanning by ribosomes or the nature of the single-stranded protruding ends of the hairpin transcript could play a role. However, although the noticeable higher concentration of siRNA produced from the hpGFP/As-S construct indicates this construct to be a stronger inducer for the production of siRNAs, such an excess does not seem to be necessary for efficient *GFP* suppression.

Transitive silencing

Using *GFP*:*GUS* and *GUS*:*GFP* hybrid constructs we have further investigated transitive RNA silencing within the agroinfiltrated zone. Under the conditions we used, the ss constructs did not induce siRNAs when infiltrated in wt plants. However, we observed spreading of silencing in both directions, since *GUS*-specific siRNAs were produced regardless of whether *GUS* sequences were located at the 5′ or 3′ side of the original *GFP* trigger. The same phenomenon has been observed in whole plants (Braunstein *et al*., 2002; Klahre *et al*., 2002; Vaistij *et al*., 2002; Van Houdt *et al*., 2003; Vanitharani *et al*., 2003), but it is remarkable that spread of silencing can be recapitulated within a short period of time in the agroinfiltrated zone. Also here we noticed a striking difference in the pattern of siRNAs. The hairpin-derived *GFP* siRNA showed again a high portion of 24-mer siRNAs, but the secondary *GUS*-specific siRNA, originating from the spread of silencing, were mostly of the small-size class (21–22-nt). This indicates that the different Dicers can discriminate between DNA-expressed ds RNA and ds RNA generated by RDR in the cytoplasm. Possibly the high levels of expression or other 'aberrant' features of transgenes may induce transitive RNAi. It has been shown that the level of expression plays an important role in susceptibility to gene silencing (Cutter *et al*., 2003) and that there is a correlation between the level of endogenous mRNA and the silencing of transgenes (a certain treshold level of endogene is required for the co-suppression of transgene and endogen sequences and for extensive silencing of the transgenes) (Han *et al*., 2004) .

In our system, in *N. benthamiana Virp*-overexpressing plant the silencing process of the endogenous sequence did not spread neither along a gene nor throughout the entire plant. Both in wt plants and the transgenic line overexpressing the 'endogenous' gene sequence of *Virp1* silencing did not spread along the *Virp1* gene. These results suggest that some inherent feature of the 'endogene' sequence may prevent spreading of silencing along the endogenous sequence. It was proposed before that certain structural features of the endogens or association with specific proteins may inhibit the RdRP which is necessary for spreading (Vaistij *et al*., 2002). It is possible that the *Virp1* gene is simply not sensitive for such a suppression, since it seems that each target sequence possesses an inherent degree of susceptibility to RNAi (Kerschen *et al*., 2004). However, tobacco β-1,3-glucanase gene served as a template for the production of secondary siRNAs (Sanders *et al*., 2002). This discrepancy could possibly be explained by inherent differences in the systems

used (*N. benthamiana* versus *N. tabacum*) (Yang *et al.,* 2004). Alternatively, it is conceivable that silencing does spread along the *Virp1* sequence but the concentration of the siRNAs produced is too low to be detected, unlike the primary siRNAs produced by the inducer construct.

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