An active role for endogenous β -1,3-glucanase genes in transgene-mediated co-suppression in tobacco

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Post-transcriptional gene silencing (PTGS) is characterized by the accumulation of short interfering RNAs that are proposed to mediate sequence-specific degradation of cognate and secondary target mRNAs. In plants, it is unclear to what extent endogenous genes contribute to this process. Here, we address the role of the endogenous target genes in transgene-mediated PTGS of β -1,3-glucanases in tobacco. We found that mRNA sequences of the endogenous glucanase *glb* gene with varying degrees of homology to the *Nicotiana plumbaginifolia* *gn1* transgene are targeted by the silencing machinery, although less efficiently than corresponding transgene regions. Importantly, we show that endogene-specific nucleotides in the *glb* sequence provide specificity to the silencing process. Consistent with this finding, small sense and antisense 21- to 23-nucleotide RNAs homologous to the endogenous *glb* gene were detected. Combined, these data demonstrate that a co-suppressed endogenous glucanase gene is involved in signal amplification and selection of homologous targets, and show that endogenous genes can actively participate in PTGS in plants. The findings are introduced as a further sophistication of the post-transcriptional silencing model.

Keywords: co-suppression/gene silencing/ β -1,3-glucanase/RNAi/tobacco

Introduction

In a wide variety of organisms, the introduction of transgenes into the genome may result in co-suppression of the transgene and homologous endogenous genes (Pal-Bhadra *et al.*, 1997; Ruiz *et al.*, 1998; Vaucheret *et al.*, 1998; Kooter *et al.*, 1999; Dernburg *et al.*, 2000; Cogoni, 2001). Many cases of co-suppression are based on sequence-specific degradation of transgene and endogenous mRNAs (Depicker and Van Montagu, 1997; Meins, 2000; Vaucheret *et al.*, 2001). This post-transcriptional gene silencing (PTGS) mechanism, originally discovered in transgenic plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990), is also capable of targeting viral RNA and is

thought to be a natural defense mechanism against viral infection in plants (Vance and Vaucheret, 2001; Voinnet, 2001; Waterhouse *et al.*, 2001). Sequence-specific RNA silencing is also observed in *Neurospora crassa* (quelling; Romano and Macino, 1992; Cogoni *et al.*, 1996), *Caenorhabditis elegans* (RNAi; Fire *et al.*, 1998), *Drosophila* (Tuschl *et al.*, 1999; Kennerdell and Carthew, 2000) and mammalian cells (Elbashir *et al.*, 2001a).

Biochemical and genetic studies conducted in the above organisms revealed striking similarities between PTGS, RNAi and quelling (Hammond *et al.*, 2001; Matzke *et al.*, 2001). RNA silencing is initiated upon production of double-stranded (ds) RNA (Fire *et al.*, 1998; Hamilton *et al.*, 1998; Waterhouse *et al.*, 1998) and correlates with the accumulation of small 21- to 23-nucleotide (nt) RNAs (Hamilton and Baulcombe, 1999; Parrish *et al.*, 2000; Zamore *et al.*, 2000). The causal relationship between dsRNA, small RNAs and target degradation was demonstrated in cell-free *Drosophila* systems, where the dsRNA trigger molecule is processed into the 21- to 23-nt short interfering RNAs (siRNAs) that guide target mRNA cleavage (Hammond *et al.*, 2000; Elbashir *et al.*, 2001b). Processing of the dsRNA requires the RNase III-related Dicer enzyme, whereas degradation of single-stranded target RNA (ssRNA) is catalyzed by the RNA-induced silencing complex (RISC) (Bernstein *et al.*, 2001). The Dicer–RISC pathway provides a basic framework to account for passive targeting of sequences homologous to the dsRNA trigger.

Additional processes at the RNA level involving RNA-dependent RNA polymerase (RdRP) activity on silencing targets have been proposed to account for signal amplification and maintenance of silencing (Dougherty and Parks, 1995; Wassenegger and Pelissier, 1998). Proteins with homology to RdRP were shown to be required for efficient silencing in fungal (Cogoni and Macino, 1999), nematode (Smardon *et al.*, 2000) and plant (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000) systems. Further support for the role of RdRPs in RNA silencing comes from studies with a *Drosophila* embryo extract demonstrating that siRNAs serve as primers to convert the target mRNA into dsRNA that subsequently generates new siRNAs (Lipardi *et al.*, 2001). Evidence for a mechanism of dsRNA synthesis, secondary siRNA amplification and subsequent degradation of new silencing targets was also obtained for *C.elegans* and referred to as transitive RNAi (Sijen *et al.*, 2001).

For plants, the contribution of endogenous genes to the post-transcriptional silencing process is still to be clarified. Early reports revealed trends that an increased expression of endogenous genes correlated with initiation or enhancement of silencing (Smith *et al.*, 1990; Seymour *et al.*, 1993; Boerjan *et al.*, 1994; Dorlhac de Borne *et al.*, 1994), which could be interpreted as arguments supporting an

Fig. 1. Endogenous *glb* sequences are targeted less efficiently by the silencing mechanism than corresponding transgene *gn1* sequences. (A) Schematic presentation of the *gn1* and *glb* mRNAs. Exons are indicated. The *glb* test regions eK and eL, and the corresponding *gn1* test regions tK and tL, are represented with solid lines. The two K regions both have a length of 351 nt, and share 77% homology with a maximal stretch of 32 nt of uninterrupted homology. The two L regions both have a length of 297 nt, and share 83% homology with a maximal stretch of 36 nt of uninterrupted homology. Plasmids carrying the test regions between the STNV leader and trailer (Jacobs *et al.*, 1999) were linearized and *in vitro* transcribed to produce chimeric viral RNAs for delivery into protoplasts. (B) 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 the test sequences shown in (A). ³²P-labeled RNA probes for detection of viral RNAs and rRNA were complementary to the (+) strand of the STNV trailer, the (+) strand of TNV and 18S rRNA sequences. For TNV and 18S rRNA probes, exposures of equal duration are shown for hemizygous and homozygous samples. For the STNV probing, a longer exposure is shown for homozygous samples as compared with hemizygous samples. (C) Relative accumulation of chimeric STNV RNAs in protoplasts of hemizygous versus homozygous plants (He/Ho ratio). In each case, the STNV signal was normalized to the 18S rRNA signal before calculation of the ratio. A higher He/Ho ratio indicates a higher silencing susceptibility. Results from two independent experiments are shown.

active role of endogenous genes in PTGS. A number of recent investigations suggest, however, that endogenous genes, unlike transgenes, do not actively participate in the

amplification and maintenance phases of the silencing process. First, the endogenous ribulose biphosphate carboxylase small subunit (*rbcS*) and phytoene desaturase (*pds*) genes seem to lack the capacity to sustain virus-induced gene silencing (VIGS) in wild-type *Nicotiana benthamiana* plants (Jones *et al.*, 1999; Voinnet *et al.*, 2000). In contrast, VIGS of a GFP transgene did progress towards autonomy after elimination of the viral trigger. Secondly, in contrast to the GFP transgene, the endogenous plant genes *rbcS* and *pds* seem to lack the capacity for secondary siRNA production upon VIGS (Vaistij *et al.*, 2002). Thirdly, graft-induced silencing of an endogenous plant gene was not maintained upon regrafting onto wild-type stocks, in contrast to graft-induced silencing of a transgene (Palauqui and Vaucheret, 1998). Together, these observations suggest that transgenes participate in the different phases of silencing, such as signal amplification and target selection, whereas endogenous genes may be mere passive targets for silencing, at least in the absence of the original silencing trigger.

To further explore the role of endogenous plant genes in post-transcriptional gene silencing, we examined whether an endogenous glucanase gene actively participates in transgene-mediated co-suppression. Silenced plants of the transgenic tobacco line T17 display co-suppression of the *Nicotiana plumbaginifolia gn1* transgene and a group of highly conserved endogenous β -1,3-glucanase genes (de Carvalho *et al.*, 1992; de Carvalho Niebel *et al.*, 1995; van Eldik *et al.*, 1998). These endogenous genes have nearly identical coding sequences and are ~81% similar to the *gn1* transgene (de Carvalho Niebel *et al.*, 1995). Here, we show that internal mRNA sequences from a member of the co-suppressed endogenous glucanase family, the *glb* gene, with a low homology to the *gn1* transgene are still targeted for silencing, although less efficiently than corresponding transgene sequences. In contrast, artificial tester sequences sharing the consensus sequence between the transgene and the endogenous *glb* gene, but with different mismatching nucleotides at non-identical positions, are not targeted by the silencing mechanism. This indicates that, in addition to the transgene, the endogenous genes provide sequence specificity to the silencing process. Consistent with this, we show that small sense and antisense RNAs specific for the endogenous glucanase genes accumulate in co-suppressed T17 plants. These data provide the first direct evidence for the involvement of endogenous plant genes in siRNA production and selection of targets for RNA silencing.

Results

Endogenous *glb* mRNA sequences are targeted by the silencing machinery, yet less efficiently than corresponding transgenic *gn1* mRNA sequences

As a first step in understanding the role of the endogenous glucanase genes in co-suppression, we compared the silencing susceptibility of sequences derived from the endogenous *glb* gene with those of corresponding transgene regions of the same length. To enable quantitative comparisons, we used a two-component viral system consisting of an autonomous helper virus (tobacco necrosis virus; TNV) encoding the viral replicase and a separate chimeric satellite virus (satellite tobacco necrosis

virus; STNV) containing the silencing test sequence. In this system, the accumulation level of chimeric STNV RNA in protoplasts of hemizygous, glucanase-expressing (He) compared with homozygous, silenced (Ho) T17 plants (He/Ho ratio) is a measure of silencing susceptibility. Using this reporter system, we previously showed that with the exception of the 5' and 3' *gnl* mRNA termini, sequences throughout the *gnl* mRNA are targets for post-transcriptional silencing. Furthermore, we showed that the silencing susceptibility of a target sequence increases with its length (Jacobs *et al.*, 1999). Chimeric STNV RNAs containing corresponding *gnl* and *glb* regions (K and L, 351 and 297 nt, respectively; Figure 1A) were co-delivered with TNV RNA to protoplasts of He and Ho T17 plants. In parallel, chimeric STNV RNAs containing silencing-insensitive 5' and 3' *gnl* mRNA sequences were included in the experiment. Twenty hours after delivery, total RNA was harvested for northern blot analysis. To verify whether differences in chimeric STNV-glucanase RNA accumulation in electroporated protoplasts were caused by variation in replicase availability, we systematically measured TNV RNA accumulation in the inoculated protoplasts. In each sample, TNV accumulated to comparable levels, implying that observed differences in STNV-glucanase RNA accumulation were due to different efficiencies of silencing (Figure 1B).

The chimeric STNV RNAs containing silencing-insensitive 5' and 3' *gnl* mRNA sequences accumulated to comparable levels in protoplasts of hemizygous and homozygous plants (data not shown), showing that protoplasts of both genotypes support viral RNA accumulation similarly.

The results in Figure 1B (top panels) show that in protoplasts of expressing (He) T17 plants, each of the chimeric STNV-glucanase RNAs accumulated to relatively high levels, whereas in protoplasts of silenced (Ho) T17 plants much lower accumulation levels were measured for all STNV-glucanase RNAs. This implies that the silencing machinery targets the K and L regions of both the transgenic and the endogenous mRNA. Importantly, comparison of the silencing susceptibilities of transgenic and endogenous K and L regions (He/Ho ratios, calculated as outlined above) shows that transgenic sequences are substantially more susceptible to silencing than the corresponding endogenous sequences (Figure 1C). In two independent experiments, the He/Ho ratio for the transgenic K region was 2.2- and 3.3-fold higher than for the corresponding endogenous sequence. Similarly, the He/Ho ratio for the transgenic L region was 3.2- and 4.2-fold higher than for the corresponding endogenous sequences. These data indicate that, for both regions, transgenic mRNA sequences are more sensitive to PTGS than the corresponding endogenous *glb* mRNA regions.

Endogene-specific nucleotides contribute to silencing efficiency

The previous experiment showed that transgenic glucanase mRNA sequences are targeted more efficiently than corresponding endogenous *glb* mRNA sequences. This could mean that PTGS in the T17 line is primarily directed against the transgene and that the endogenous glucanase mRNAs are passively silenced by transgene-derived mediators of silencing-related RNA degradation, such as

the 21- to 23-nt RNAs described by Hamilton and Baulcombe (1999). In this view, 'cross'-silencing of endogenous mRNAs would be less efficient than silencing of transgene mRNAs as the co-suppressed endogenous mRNAs would not allow perfect pairing with transgene-derived mediators over most of their sequence. Alternatively, the lower silencing susceptibility of endogenous compared with transgenic mRNA sequences could relate to a difference in abundance of transgene- and endogene-specific mediators. This would imply that the co-suppressed endogenous genes acquire an active role in the silencing process through the production of endogene-specific mediators at some stage after initiation.

To distinguish between the above two scenarios, we set up a functional assay to examine whether the silencing susceptibility of a certain test sequence is determined exclusively by the homology level (or similarity) to the transgene or whether endogene-specific nucleotides at non-identical positions contribute to the silencing efficiency of these sequences. To verify the silencing effect of random nucleotide sequences in these non-identical positions, we determined the silencing efficiency of artificial tester sequences that correspond to the consensus sequence between the transgene and the endogenous genes but lack homology to both sequences at non-identical positions (Figure 2A).

We selected for this purpose three 65 nt regions of the *gnl* and *glb* mRNA (X, Y and Z; Figure 2A). In these regions, the homologies between the transgenic, the endogenous *glb* and the artificial tester sequences are 58, 74 and 89%, respectively (Figure 2B).

We reasoned that if the co-suppressed endogenous genes have merely a passive role in PTGS, endogenous and artificial tester sequences with similar homology to the transgene will in all instances be equally susceptible to silencing. Furthermore, in this scenario, endogenous and artificial tester sequences corresponding to the X region are unlikely to be recognized by the silencing mechanism as the overall homology to the *gnl* transgene is only 58% and the largest stretch of perfect homology is only 8 nt. Alternatively, if endogene-specific mediators of RNA silencing were to exist in silenced plants, endogenous mRNA sequences should be targeted consistently more efficiently than the corresponding artificial tester sequences. Furthermore, even the low-homology endogenous X region (eX) would be expected to be susceptible to PTGS.

Chimeric STNV RNAs containing the transgenic, endogenous and artificial glucanase tester sequences were co-delivered with wild-type TNV RNA to protoplasts of expressing and silenced T17 plants, and viral RNA accumulation was determined 20 h after inoculation (Figure 2C). TNV accumulated to comparable levels in all samples (data not shown).

For each region tested, we observed the strongest silencing effect for STNV RNAs containing transgenic sequences (Figure 2D). This confirms the previous observation that transgenic mRNA regions are more susceptible to PTGS than endogenous *glb* sequences. The data also show that the silencing machinery recognizes the 'high'-homology artificial tester region Z (aZ), indicating that an overall homology of 89% and/or a region of 32 nt perfect homology to the co-suppressed genes is sufficient to carry

Fig. 2. Endogene-specific nucleotides contribute to sequence specificity of the RNA degradation step of PTGS. (A) Upper part: position of the X, Y and Z test subregions within the K test region. Lower part: display of the homology between the *glb*, *gn1* and artificial test sequences in the X, Y and Z subregions. The gray boxes show the 'consensus sequence' between the transgenic, endogenous and artificial test sequences. Plasmids carrying the test regions between the STNV leader and trailer (Jacobs *et al.*, 1999) were linearized and *in vitro* transcribed to produce chimeric viral RNAs for delivery into protoplasts. (B) Features of the subregions X, Y and Z. The length of the X, Y and Z test sequences is shown, as well as the overall similarity between endogenous, transgenic and artificial sequences. For each subregion, the largest stretch of uninterrupted homology between the endogenous, transgenic and artificial sequences is shown. (C) Northern blot analysis of total RNA extracted from protoplasts of He and Ho T17 plants 20 h after delivery of TNV RNA and chimeric STNV RNA containing transgenic, endogenous and artificial test sequences as shown in (A). The 32 P-labeled RNA probe was complementary to the (+) strand of the STNV trailer. (D) Relative accumulation of chimeric STNV RNAs in protoplasts of hemizygous versus homozygous plants (He/Ho ratio). Bars represent the average of two (Z region) or three (X and Y region) independent experiments. CAT: STNV-CAT; the He/Ho ratio for the silencing-insensitive STNV CAT RNA is indicated by the horizontal dashed line. Letter codes above columns indicate statistical significance of the differences between silencing susceptibilities within each region, as determined by repeated measures ANOVA. Columns marked a, b, c differ significantly at 95% confidence. The column marked a/b differs from those marked a and b at 88 and 80% confidence, respectively.

a sequence into PTGS via cross-silencing. This is not surprising taking into account that RNA molecules as short as 21–23 nt can mediate degradation of homologous target RNA *in vitro* and in intact plants (Elbashir *et al.*, 2001b; Thomas *et al.*, 2001), implying that a target size of well under 32 nt can be sufficient.

Importantly, the experiments show that chimeric STNV RNAs carrying the endogenous 'low'- and 'medium'-homology regions X and Y were silenced, whereas chimeric STNV RNAs containing the corresponding artificial tester regions X and Y were not. The silencing insensitivity of artificial tester sequences in regions X and Y indicates that silencing of the endogenous X and Y sequences was not due to cross-silencing via transgene-specific mediators, and thus implies that these endogenous regions are recognized by endogene-specific siRNAs. In other words, the data indicate that, in addition to the transgene, the endogenous genes provide specificity to the silencing process.

Small sense and antisense RNAs specific for the co-suppressed endogenous genes accumulate in silenced T17 plants

Following current silencing models, participation of the endogenous glucanase genes in the sequence-specific RNA degradation step of PTGS would involve the production of endogene-specific siRNAs. We therefore set out to detect such small RNAs in protoplasts of non-infected, silenced T17 plants.

As a first step, we examined whether PTGS in the T17 line correlates with the presence of small 21- to 23-nt RNAs and whether these silencing molecules accumulate in protoplasts of silenced (Ho) T17 plants. To this end, polyethylene glycol (PEG)-enriched low-molecular-weight RNA–DNA fractions from protoplasts and leaf tissue of silenced (Ho) and expressing (He) T17 plants were separated on a 15% polyacrylamide gel, blotted onto a nylon membrane and hybridized with riboprobes corresponding to nearly the entire *gn1* mRNA region

Fig. 3. Glucanase silencing correlates with accumulation of small sense and antisense RNAs homologous to *gnl*. (A) Nucleic acids from protoplasts and leaf tissue of hemizygous, expressing and homozygous, silenced T17 plants were enriched by PEG. Samples of 10 µg were separated on a 15% polyacrylamide gel and blotted to membranes. Filters were hybridized with ³²P-labeled RNA probes corresponding to the (+) or (–) strand of the *gnl* cDNA. The arrows indicate the position of the small RNA species. Comparison to RNA size markers of 20, 22, 25 and 28 nt indicates that the small RNA species have a length of 21 and 23 nt. He, hemizygous, expressing plants; Ho, homozygous, silenced plants; pps, leaf protoplasts; tiss, leaf tissue.

(see Materials and methods). The results in Figure 3 show that small sense and antisense glucanase oligonucleotides accumulate in protoplasts and leaf tissue of silenced (Ho) T17 plants, but not in protoplasts and tissue of expressing (He) T17 plants. RNase and DNase treatment of the low-molecular-weight fraction confirmed that the small nucleotides accumulating in silenced T17 plants are ribonucleic acids (data not shown). Taken together, the results show that silencing in the T17 line is associated with the presence of 21- to –23-nt glucanase RNAs and that these molecules accumulate to similar levels in protoplasts and tissue of silenced plants.

To detect endogene- and transgene-specific small RNAs in protoplasts of silenced plants, we probed for small RNAs corresponding to region X of *gnl* and *glb* (Figure 2A). This region was chosen because the degree of homology between endogenous and transgenic X sequences is expected to be sufficiently low (58%) for specific detection of endogene- and transgene-derived small RNAs in hybridization experiments.

Equal amounts of PEG-enriched small RNA fractions from protoplasts of silenced and expressing T17 plants were run on polyacrylamide gels, blotted onto nylon membranes and hybridized with four RNA probes for detection of endogene- and transgene-specific small sense and antisense RNAs corresponding to the X region. Control hybridizations with *in vitro* synthesized endogenous and transgene sequences corresponding to region X demonstrated that no cross-hybridization occurred. Therefore, any signal generated by each of the four probes was specific for the endogenous or transgenic sequences in either orientation (Figure 4).

Small RNAs were detected in protoplasts of silenced plants with all four probes. This demonstrates that small sense and antisense RNAs originating from the transgene

and the endogenous glucanase genes co-exist in protoplasts of silenced T17 plants. This implies that a co-suppressed endogenous glucanase gene is involved in synthesis of the siRNA signal. Interestingly, quantification relative to *in vitro* synthesized control RNAs showed that endogene-specific guide RNAs corresponding to region X are ~3- to 10-fold more abundant than the corresponding transgene-specific RNAs (data not shown). This is consistent with the observation that transgenic mRNAs are better targets for PTGS than endogenous mRNAs.

In conclusion, the siRNA detection experiments and the functional assays indicate that, in the presence of the *gnl* transgene, an endogenous glucanase gene is activated for production of siRNAs, leading to a more active silencing than would occur in a scenario of mere passive involvement.

Discussion

In the present study, we have investigated whether endogenous target genes actively participate in transgene-mediated co-suppression. Several studies in plants suggest that whereas transgenes can become actively involved in setting up and maintaining a silenced state, endogenous genes may be merely passive targets for the silencing mechanism. In this paper, we provide the first direct evidence that an endogenous target gene is actively involved in the silencing process of transgene-mediated co-suppression in plants. The detection of endogene-specific small sense and antisense RNAs, and the preferential suppression of the accumulation of chimeric STNV RNAs containing these endogene-specific sequences in protoplasts of silenced T17 plants, indicate that a co-suppressed endogenous glucanase gene is a template for small RNA synthesis and thereby contributes to selection of silencing targets.

In principle, the small endogene-specific sense RNAs we observed in silenced plants could be products either of endogenous mRNA degradation or of active synthesis. However, biochemical evidence indicates that degradation of single-stranded silencing target RNA does not lead to the formation of the characteristic small siRNAs (Elbashir *et al.*, 2001b). Furthermore, passive glucanase mRNA degradation does not explain the accumulation of small antisense RNAs. We therefore conclude that the endogene-specific small sense and antisense RNAs derive from a process that involves the synthesis of antisense RNA molecules on a sense template. Lipardi *et al.* (2001) have recently provided evidence for a relay-amplification mechanism in which primary siRNAs prime the synthesis of dsRNA, leading to production of secondary siRNAs. This mechanism is proposed to be responsible for the transition of virus-induced transgene silencing into a virus-independent maintenance stage in plants (Jones *et al.*, 1999; Voinnet *et al.*, 2000) and for spreading of the silencing target spectrum or transitive RNAi in nematodes (Sijen *et al.*, 2001; Vaistij *et al.*, 2002). The observation that in protoplasts of silenced T17 plants both endogene- and transgene-specific small RNAs accumulate is consistent with a process of secondary siRNA production. The viral RNA delivery experiments performed in this study demonstrate a functional consequence thereof for silencing efficiency. Therefore, we propose that in T17 plants

Fig. 4. Transgene- and endogene-specific small RNAs accumulate in protoplasts of silenced T17 plants. Nucleic acids from protoplasts and leaf tissue of hemizygous, expressing and homozygous, silenced T17 plants were enriched by PEG. Samples of 45 μ g and *in vitro* synthesized control RNAs were separated on a 15% polyacrylamide gel, blotted onto membranes and hybridized with 32 P-labeled RNA probes corresponding to the (+) or (–) strand of transgenic and endogenous X region. The arrowheads indicate the position of the small RNA. Control RNAs: sense tX, sense eX; *in vitro* synthesized sense RNAs of ~70 nt corresponding to transgenic or endogenous region X; antisense tX, antisense eX; *in vitro* synthesized antisense RNAs of ~70 nt, corresponding to transgenic and endogenous region X. Probes for eX detection do not cross-hybridize to *in vitro* synthesized tX RNA and vice versa.

spreading of RNA targeting (transitive PTGS) between transgenes and endogenous genes takes place and, in more general terms, that this mechanism contributes to the specificity and efficiency of gene silencing in plants.

At this stage, it is unclear how general are our findings. Several observations have suggested that the transitivity process and the underlying mechanisms do not apply to endogenous plant genes. It was shown, for example, for two endogenous plant genes that VIGS does not progress into a virus-free maintenance phase and is not associated with production of endogenous siRNAs and spreading of RNA targeting (Jones *et al.*, 1999; Voinnet *et al.*, 2000; Vaistij *et al.*, 2002). This raises the question why in the T17 line a co-suppressed endogenous target gene participates in siRNA production. First, it is possible that transgenes are more effective than viral RNAs in recruiting endogenous genes into an active role in silencing. This could be the case if recruitment of endogenous genes into an active role requires a nuclear phase, which the viral inducers may be less able to provide. Secondly, it is possible that some endogenous genes are more susceptible to transitivity than others. This could relate to a range of factors, among which are the chromatin status, the RNA expression level, the capacity to form specific secondary structures or the presence of specific recognition elements, for example. More generally, this could also apply to transgenes. The observation that, in accordance to what is observed for endogenous genes, silencing of transgenes does not by default lead to spreading of RNA targeting (English *et al.*, 1996; Elmayan *et al.*, 1998; Wang *et al.*, 2001) supports the idea that there is no principal distinction between transgenes and endogenous genes with respect to their ability for recruitment into an active role in RNA silencing.

Based on our results and in line with the model for transitive RNAi (Sijen *et al.*, 2001), we propose a model for the communication between inducing transgene and endogenous target genes in plants in which the endogenous genes adopt an active role in PTGS via a process involving four major steps (Figure 5): (a) interaction between transgene-derived siRNA and endogenous *glb* mRNA; (b) RdRP-mediated complementary RNA synthesis; (c) Dicer-mediated production of *glb*-derived siRNA; and (d) targeting of endogenous mRNA by cognate siRNA. The model presupposes that the transgene is the first to produce siRNAs, as silencing is frequently observed upon introduction of (complex) transgene sequences. The proposed steps require functional RdRP and Dicer-like activities, but do not depend on the RISC component, which is still under debate for plants.

In another case of transgene-induced silencing, Han and Grierson (2002) detected small RNAs corresponding to the 5' end of the co-suppressed genes, but could not distinguish whether these are of endogenous or transgenic origin. They did not detect small RNAs corresponding to the endogene-specific 3' mRNA region of the co-suppressed endogenous gene. In this silencing case, the transgene is truncated at the 3' end and has no homology to the 3' part of the endogenous gene. The findings are consistent with the above model under the assumptions that (i) the 5' small RNAs are at least partly of endogenous origin and (ii) secondary siRNA production is directional and proceeds in a 3' to 5' direction relative to the template mRNA.

For the T17 silencing case, we observed that *gn1* and *glb* leader sequences are not targeted by a silencing mechanism (Jacobs *et al.*, 1999; our unpublished data). This is surprising, as the RdRP-mediated relay-amplification

Fig. 5. Model explaining the activation of endogenous genes in PTGS. It assumes that transgene-specific (light gray) and 'common' (black) siRNAs are produced from the inducing transgene. The small transgene-derived 'common' antisense siRNAs anneal to homologous regions (black boxes) in the endogenous mRNA (a). The resulting partial dsRNA-RNA hybrids are recognized by an RNA-dependent RNA polymerase (RdRP) for elongation (b) or by the RNA-induced silencing complex (RISC) for direct degradation (dashed arrow). The dsRNA molecules resulting from RdRP activity are processed further by a Dicer-like enzyme, leading to the accumulation of secondary, endogene-derived siRNAs, the sequence of which can be endogene specific (dark gray) or common (black) (c). The endogene-specific secondary siRNAs can directly tag secondary targets such as endogene-specific regions in the endogenous mRNA (d), which subsequently become substrates for RISC-related degradation or a second round of RdRP-mediated dsRNA production and Dicer cleavage (dashed arrows).

reactions on mRNA templates predict the synthesis of siRNAs corresponding to 5' mRNA termini. In view of the model, the absence of such 5' siRNAs could mean either that the 5'-termini of glucanase mRNA are protected against RdRP-dependent synthesis of antisense RNA or that these sequences are protected from Dicer-mediated degradation.

In the model, we propose that communication between the transgene and the homologous endogenous genes occurs at the RNA level. However, this does not preclude the possibility that in this or other silencing cases additional DNA-DNA or RNA-DNA interactions may be involved. For instance, the observations that transitivity occurred following bombardment with promoterless DNA constructs in *N.benthamiana* (Voinnet *et al.*, 1998) and that transitivity was associated with the activity of Polycomb type chromatin binding proteins in *Drosophila* (Pal-Bhadra *et al.*, 1999) suggest the existence of additional target spreading mechanisms at the DNA level.

The importance of PTGS in natural gene regulation is not clear, although there are some indications that PTGS and RNAi are genetically linked with developmental control (Matzke *et al.*, 2001). Such a role would (at least) require that endogenous genes are capable of producing mediators of sequence-specific RNA degradation. The observation that in silenced T17 plants the endogenous glucanase genes are involved in production of siRNAs and silencing target selection adds evidence to the idea that post-transcriptional silencing driven by and directed against endogenous genes is part of the natural repertoire of mechanisms to regulate gene expression in plants.

Materials and methods

Plant material and growth conditions

Homozygous and hemizygous T17 plants (transgenic *Nicotiana tabacum* cv Petit Havana SR1; de Carvalho *et al.*, 1992) and untransformed SR1 tobacco plants were germinated and grown *in vitro* on solid medium in a growth chamber (25°C, 70 µmol/m²/s light intensity, 14 h light:10 h dark period). Plants were propagated via cuttings every 7–9 weeks.

Plasmid constructions

The construction of plasmids pTNV-A and pSTNV-2 containing full-length cDNA copies of the TNV-A and STNV-2 genomes, respectively, and permitting synthesis of infectious *in vitro* transcripts, is described elsewhere (Andriessen, 1997). The construction of pKB11 from pSTNV-2 is described in Andriessen (1997). In this construct, the distal 456 nt of the STNV CP and the first 105 nt of the STNV-2 trailer are replaced by the *npt II* coding region. The construction of plasmids pSTNV-tK and pSTNV-tL is described in Jacobs *et al.* (1999) (plasmids -tk and -tl are identical to pSTNV-K and pSTNV-L). Plasmids pSTNV-eK, pSTNV-eL and pSTNV-eM were synthesized from pKB11 by replacing a *HindIII*–*SalI* fragment containing the *npt II* coding region by fragments covering the selected sequences. The inserted fragments were obtained by PCR on the pglb3 plasmid. Plasmids pSTNV-tX to pSTNV-aZ were constructed from pKB11 by replacing the *HindIII*–*SalI* fragment by synthetic dsDNA oligonucleotides spanning the selected regions. The sequence of artificial tester oligonucleotides (aX, aY and aZ) was designed such that all conserved nucleotides between *gnl* and *glb* were maintained, while at positions where the sequence diverged, a random nucleotide was introduced that was different from those present in the *gnl* and *glb* sequence. The construction of pMA491 (pSTNV-CAT) is described in Andriessen (1997).

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. The resulting transcript of 3684 nt, differing from the natural RNA only by the substitution of the 5'-terminal A by a G residue, is capable of autonomous replication in tobacco protoplasts and supports STNV replication (Andriessen, 1997). All chimeric STNV-glucanase RNAs were synthesized *in vitro* from the appropriate pSTNV plasmids linearized at the *BamHI* site, using T7 RNA polymerase. The resulting transcripts comprise the 32 nt STNV leader (except for the first nucleotide, which is G instead of A), followed by 141 nt encoding the N-terminal part of the STNV coat protein, fused with the selected test sequences and followed by the STNV-2 trailer. In all chimeric STNV RNAs, a 3'-terminal extension of 7 nt is added to the trailer, relative to wild-type STNV-2. Similar chimeric STNV RNAs containing different test sequences were previously shown to be biologically active (Jacobs *et al.*, 1999). 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 were assessed by denaturing agarose gel electrophoresis of RNA samples.

Protoplast preparation and electroporation experiments

Protoplasts were prepared from the top leaves of 7- to 9-week-old plants grown from cuttings, as described by De Block *et al.* (1987). Protoplasts were electroporated as described by Meulewaeter *et al.* (1992). Per 1×10^6 protoplasts, 0.2 pmol of TNV-A RNA and 2 pmol of chimeric STNV-2 RNA were used as an inoculum. 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 $0.5\text{--}1 \times 10^6/\text{ml}$ in 5 ml of incubation medium, in the dark at 24°C. Twenty hours after electroporation, dead cells were removed by centrifugation at 80 g, and RNA was extracted from the surviving (floating) protoplasts.

RNA isolation and analysis

RNA was extracted from protoplasts as described by Jones *et al.* (1985). Total RNA (1 µg per lane) was electrophoresed in 1.5% agarose-formaldehyde gels according to Sambrook *et al.* (1989). To verify RNA quality and quantity, ethidium bromide (0.25 µg per RNA sample) was added to samples before denaturing at 65°C. After electrophoresis, gels were blotted onto Hybond-N+ membranes (Amersham) by capillary transfer in 20× SSC. The RNA was fixed on the membranes by baking. Riboprobes to detect sense TNV, sense STNV trailer and 18S rRNA sequences were prepared from appropriate templates using a Promega

in vitro transcription kit. Blots were hybridized overnight at 68°C in 50% formamide, 10% dextran sulfate, 5× SSC, 1% SDS, 100 µg/ml denatured sheared herring sperm DNA. Filters were washed at least four times at 65°C, with the final wash in 0.1× SSC, 0.1% SDS. Signals were quantified using a Molecular Dynamics PhosphorImager and the Imagequant software.

To detect the small 21- to 23-nt RNAs, an adapted version of the Hamilton and Baulcombe (1999) 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% PEG (mol. wt 8000) and 0.5 M NaCl. The low-molecular-weight fraction was separated by electrophoresis through 15% polyacrylamide-7 M urea-0.5× Tris-borate-EDTA gels, transferred onto Hybond N+ filters (Amersham) and fixed by UV cross-linking. The filters were pre-hybridized in 45% formamide, 7% SDS, 0.3 M NaCl, 0.05 M Na₂HPO₄/NaH₂PO₄ pH 7, 1× Denhardt's solution and denatured herring sperm DNA (100 mg/ml). Hybridization was in the same solution using hydrolyzed α -³²P-labeled RNA probes corresponding to the (+) or (−) strand of the *gnI* cDNA or the X-region of *gnI* or *glb*, with an average length of 50 nt. Hybridized filters were washed twice 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 an 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.

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Note added in proof

Recently, endogenous small RNAs with typical characteristics of siRNAs have been cloned from *Arabidopsis* inflorescence tissues (Llave *et al.*, 2002). This provides further support for a potential role of sequence-specific RNA silencing in endogenous plant gene regulation.

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