The Frequency and Efficiency of Endogene Suppression by Transitive Silencing Signals Is Influenced by the Length of Sequence Homology[1][W]

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Transitivity, the spread of RNA silencing along primary target sequences, leads to the degradation of secondary targets that have no sequence homology to the initial silencing trigger. We demonstrate that increasing the distance between direct and adjacent target sequences in a transgenic primary target delays the onset of silencing of a secondary target gene. Silencing can spread in a 3′ to 5′ direction over a distance of at least 500 nucleotides (nt), but this requires consistently more time compared to a distance of 98 nt or 250 nt. The efficiency and frequency of transitive silencing of an endogene depends on the length of its sequence homology with the primary target. With a length of 500 nt, efficient silencing can eventually be established in all plants, whereas lengths of 250 nt and 98 nt homology result in less efficient and less frequent suppression. These results suggest that amplification of secondary small interfering RNAs (siRNAs) is a time-requiring process that gradually expands the population of siRNAs until a steady-state level is reached. Moreover, the length of the sequence homology in the primary target providing secondary siRNAs determines whether this steady-state level readily exceeds the threshold necessary for efficient silencing.

A wide variety of eukaryotic organisms, including plants, animals, and fungi, have developed several RNA-silencing pathways to protect their cells and genomes against invading nucleic acids, such as viruses or transposons. The RNA-silencing pathways also regulate gene expression during development or in response to external stimuli (for review, see Baulcombe, 2005; Meins et al., 2005). All RNA-silencing systems involve the processing of double-stranded RNA (dsRNA) into small RNAs (sRNAs) of 21 to 25 nucleotides (nt) by an RNaseIII-like enzyme, known as Dicer or Dicer-like (Bernstein et al., 2001; Xie et al., 2004, 2005; Dunoyer et al., 2005). These sRNAs are incorporated into silencing effector complexes containing an Argonaute protein (for review, see Meister and Tuschl, 2004). Variations on this core mechanism include different origins of the silencing trigger and different types of effector complexes. Endogenous, viral, or transgenic RNA molecules can give rise to dsRNA by fold-back of inverted repeat (IR) sequences, by hybridization of sense and antisense sequences, or by the action of an RNA-dependent RNA polymerase (RDR). Cleavage of the dsRNAs leads to the formation of transgenic and viral small interfering RNAs (siRNAs), endogenous microRNAs, trans-acting siRNAs, or chromatin-associated siRNAs. Depending on the dsRNA source, the sRNAs are recruited into different effector complexes (RNA-induced silencing complex and RNA-induced initiation of transcriptional silencing complex), resulting in sequence-specific RNA degradation, translational repression, or chromatin modifications (Béclin et al., 2002; Zilberman et al., 2003; Baumberger and Baulcombe, 2005).

Although the basic mechanism for RNA silencing is evolutionarily conserved, only nematodes, fungi, and plants have developed an RDR-dependent amplification system ensuring a robust RNA-silencing response that can expand and spread in the organism (Sijen et al., 2001; Martens et al., 2002; Forrest et al., 2004). Amplification involves the production of dsRNA by the action of an RDR, using primary target RNAs as a template. The newly synthesized dsRNA is subsequently cleaved into siRNAs that are able to guide the degradation of additional secondary target RNAs. This mechanism also enables cells to respond to silencing signals that travel throughout the organism, resulting in systemic silencing (Klahre et al., 2002; Alder et al., 2003; Himber et al., 2003; García-Pérez et al., 2004; Schwach et al., 2005). A further characteristic of RDR-mediated amplification is the production of secondary siRNAs from regions outside of the sequence initially targeted by trigger-derived primary siRNAs, termed transitivity (Sijen et al., 2001). Transitive silencing was first described in *Caenorhabditis elegans*, where it proceeds over a distance of a few hundred

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nucleotides in the 3′ to 5′ direction only through dsRNA synthesis primed by primary siRNAs (Sijen et al., 2001). In this organism, spreading of silencing is not limited to transgenic targets, but endogenous transcripts can also be used as template for amplification (Sijen et al., 2001; Alder et al., 2003). In plants, transitive silencing can occur in both the 3′ to 5′ and the 5′ to 3′ direction along transgenic target RNAs in a primer-dependent or primer-independent manner (Braunstein et al., 2002; Vaistij et al., 2002; Himber et al., 2003; Van Houdt et al., 2003; Kościańska et al., 2005; Miki et al., 2005; Petersen and Albrechtsen, 2005). Virus-induced gene silencing has been shown to spread over a distance of at least 1,000 nt from the 5′ end to the 3′ end of the target mRNA, while 3′ to 5′ spreading can extend at least through 332 nt, with a possible limit of 600 nt (Vaistij et al., 2002; Petersen and Albrechtsen, 2005). In plants, many studies failed to demonstrate transitivity along endogenous transcripts, suggesting that endogenous sequences are protected from transitivity by some inherent feature (Vaistij et al., 2002; Himber et al., 2003; Kościańska et al., 2005; Miki et al., 2005; Petersen and Albrechtsen, 2005). Indeed, only one report showed the involvement of endogenous transcripts in signal amplification and selection of homologous targets (Sanders et al., 2002).

In an XYZ system in tobacco (*Nicotiana tabacum*), we have shown that transitive amplification products that arise from silenced targets containing transgenic β-glucuronidase (*gus*) sequences are able to trigger silencing of a *gus* transgene (Van Houdt et al., 2003). We have now implemented this system in Arabidopsis (*Arabidopsis thaliana*) and showed that both transgenes and endogenes can be silenced by the secondary transitive signals (Bleys et al., 2006a). In this XYZ system, the primary target Y harbors a direct target region homologous to the silencing inducer X and an adjacent region homologous to the secondary target Z. Here, we investigated the effect on the timing of transitive silencing of increasing the distance in the primary target between the homologies with the silencing inducer and the secondary target. We also analyzed the influence on the frequency and efficiency of transitive silencing of increased length of sequence homology. We used three primary target Y constructs in which different lengths of the catalase 2 (*CAT2*) sequence were inserted between the 3′ end targeted by the silencing inducer X and the upstream region homologous to a *gus* target Z. We assessed the occurrence of transitive silencing of both secondary targets *CAT* and *gus*, looked at the relationship between silencing efficiency and secondary siRNA production, and checked the accumulation of tertiary endogenous siRNAs.

RESULTS

Increasing the Distance between the Direct Target Region and Adjacent Sequences in a Transgenic Primary Target Delays the Onset of Transitive *gus* Silencing

In tobacco, a posttranscriptionally silenced IR of a T-DNA with a highly transcribed transgene, designated as locus X, can trigger transitive silencing of a secondary target Z when a stepwise homology is created through the introduction of a chimeric primary target Y with homology to both the silencing inducer X and the secondary target Z (Van Houdt et al., 2003). Similarly, in Arabidopsis, locus X_{21} (Fig. 1; IR of P35S-*nptII*-3′*chs*) can transmit its silencing-inducing capacity to the primary target Y_7 (Fig. 1; P35S-*gus*-*nptII*-3′*chs*), which in turn is able to silence the transgenic *gus* target Z_c (Fig. 1; P35S-*gus*-3′*nos*; Bleys et al., 2006a). To assess whether the frequency of transitive *gus* silencing varies with an increasing distance between the direct and adjacent target sequences located in the primary target Y, we constructed three primary targets: Y_{98}, Y_{250}, and Y_{500}. For this purpose, we used construct Y_7, in which we inserted 98 nt, 250 nt, and 500 nt of the *CAT2*-coding sequence between the *nptII*-3′*chs* region initially targeted by the silencing trigger X_{21} and the full-length *gus*-coding sequence homologous to the secondary target Z_c (Fig. 1). Plants harboring different combinations of locus X_{21}, one of the four loci Y_* (Y_7, Y_{98}, Y_{250}, or Y_{500}), and Z_c were obtained after floral dip transformation and crossing experiments (see ''Materials and Methods'').

The GUS activity was first measured at 5 weeks in leaf protein extracts from hybrid Y_*Z_c and $X_{21}Y_*Z_c$ plants hemizygous for the present loci. All 38 $Y_{98}Z_c$, 40 $Y_{250}Z_c$, and 29 $Y_{500}Z_c$ plants showed high GUS activity (Supplemental Fig. S1), so we can conclude that none of the Y_* constructs induces in cis- or in trans-silencing of the *gus* genes in the absence of locus X_{21}. The criterion for discriminating silenced from non-silenced $X_{21}Y_*Z_c$ plants was a GUS activity value that was less than 10% of the average GUS activity of the corresponding Y_*Z_c plants. A total of 37 out of 40 (93%) $X_{21}Y_{98}Z_c$ plants, 30 out of 40 (75%) $X_{21}Y_{250}Z_c$ plants, and 12 out of 30 (40%) $X_{21}Y_{500}Z_c$ plants showed efficient *gus* silencing (Supplemental Fig. S1), indicating that the frequency of *gus* silencing at 5 weeks decreased as a function of the length of the *CAT2* insert.

Then, to confirm this result and to follow the establishment of *gus* silencing through time, we measured the GUS activity in progeny plants of self-fertilized Y_*Z_c and $X_{21}Y_*Z_c$ plants for each Y_* locus at 5, 6, and 8 weeks after sowing (see ''Materials and Methods''; Fig. 2). All Y_*Z_c plants (five plants for every Y_* construct) had a high GUS activity (Fig. 2, E–H), excluding in cis- or in trans-silencing of the *gus* genes in either hemizygous or homozygous conditions of both Y_* and Z_c loci. All 15 $X_{21}Y_7Z_c$ plants (Fig. 2D) exhibited very efficient *gus* silencing that was already established at 5 weeks. This result is in contrast to those obtained for 15 $X_{21}Y_{98}Z_c$, 30 $X_{21}Y_{250}Z_c$, and 15 $X_{21}Y_{500}Z_c$ plants (Fig. 2, A−C, respectively), which at 5 weeks had a variable frequency of silencing (80%, 77%, and 67%, respectively). After 6 weeks, more plants became silenced (93%, 83%, and 80%, respectively), and, after 8 weeks, all plants had suppressed the *gus* genes. In plants that were not silenced based on the criterion, the GUS activity ranged from 14% to 96% of the average GUS

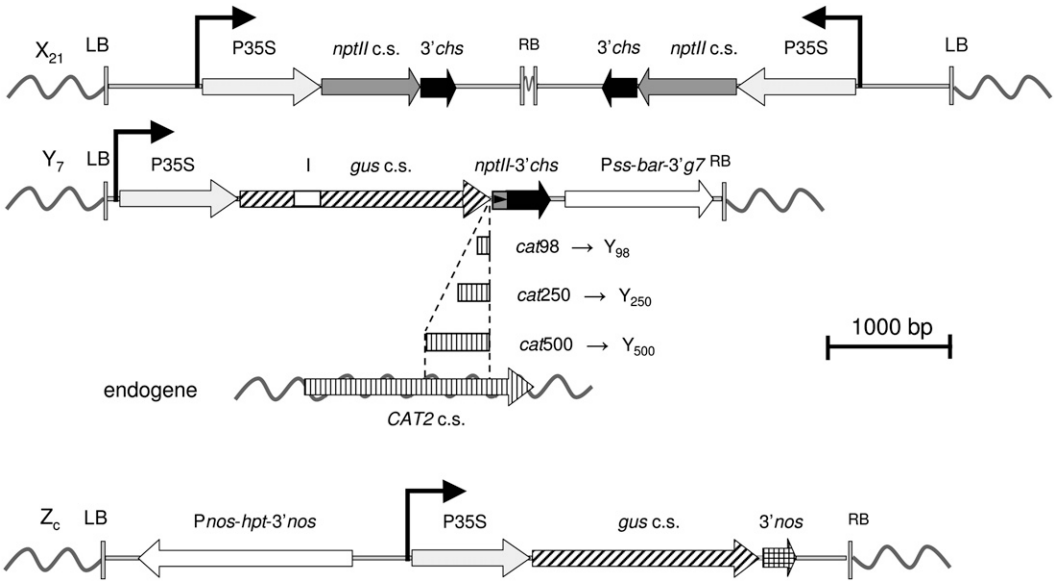


Figure 1. Schematic outline of the T-DNA constructs (drawn to scale), present in locus X_{21}, primary targets Y_7, Y_{98}, Y_{250}, and Y_{500}, and secondary target Z_c. The silencing-inducing locus X_{21} contains two T-DNA copies arranged as an IR around the right border. The loci Y_{98}, Y_{250}, and Y_{500} and locus Z_c are single-copy loci. The sequences that are inserted into Y_7 between the *nptII*-3′*chs* region targeted by the silencing trigger X_{21} and the full-length *gus*-coding sequence (*gus* c.s.) homologous to secondary target Z_c, are homologous to the 3′ part of the Arabidopsis *CAT2*-coding sequence (*CAT2* c.s.). As indicated on the scheme, the inserts overlap each other and are 98 nt, 250 nt, and 500 nt in length (*cat*98, *cat*250, and *cat*500), giving rise to Y_{98}, Y_{250}, and Y_{500}. The wavy lines represent plant DNA. 3′*chs*, 3′-untranslated region (UTR) of the chalcone synthase gene of *Anthirrinum majus*; 3′*g7*, 3′-UTR of the *Agrobacterium tumefaciens* octopine T-DNA gene 7; 3′*nos*, 3′-UTR of the nopaline synthase gene; *bar*, bialaphos acetyltransferase-coding sequence conferring phosphinothricin resistance; *hpt*, hygromycin phosphotransferase-coding sequence; I, artificial intron; *nptII* c.s., neomycin phosphotransferase II-coding sequence; P35S, cauliflower mosaic virus 35S promoter; P*nos*, nopaline synthase promoter; P*ss*, promoter of the small subunit of Rubisco; LB, left T-DNA border; RB, right T-DNA border.

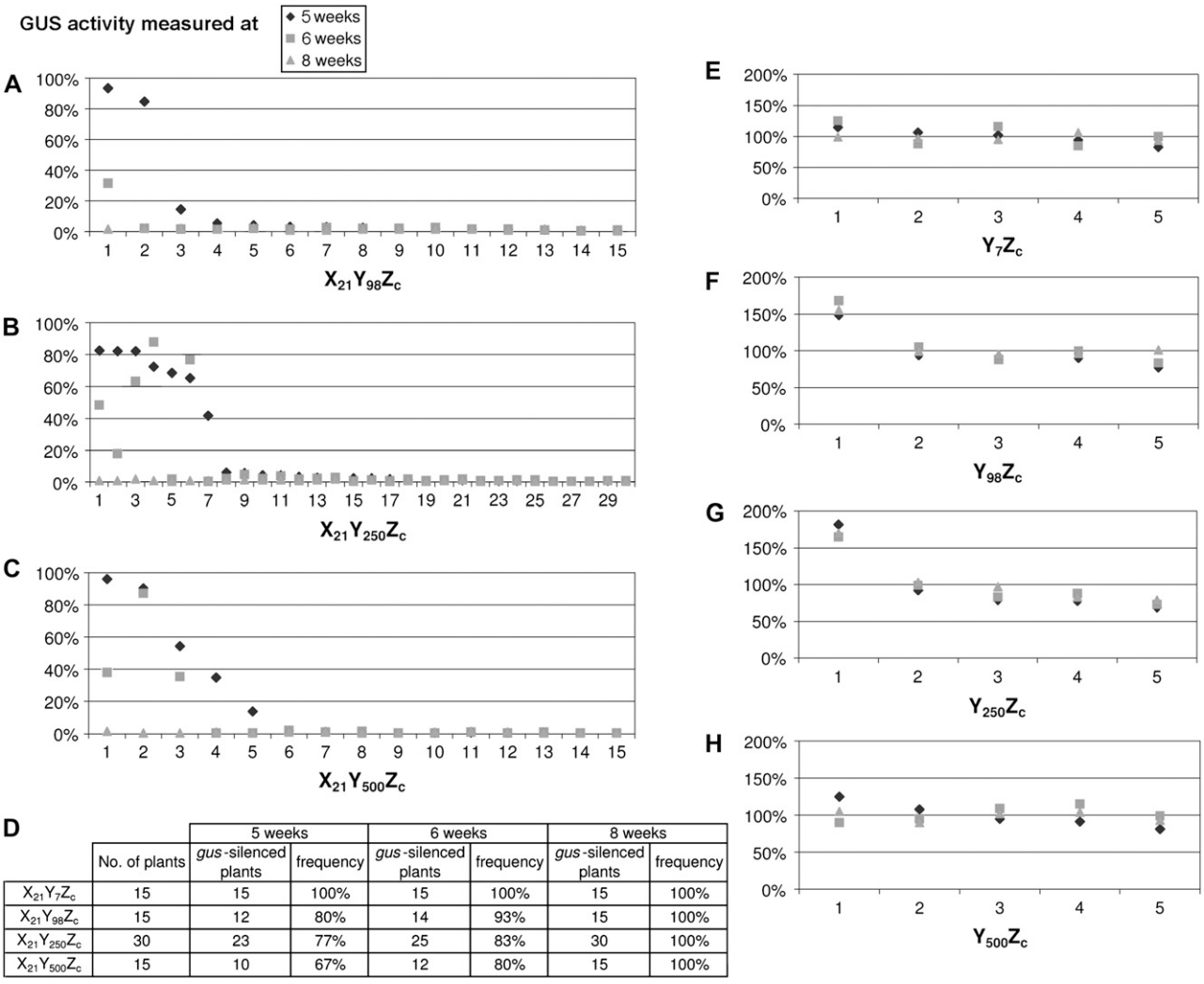
activity of the corresponding Y_*Z_c plants (Fig. 2, A–C). The occurrence of different degrees of silencing (referred to as silencing efficiency) indicates that transitive silencing was induced gradually or that in some plant cells silencing was complete, while in others it was not established yet. From these data, we can conclude that the onset of silencing of Z_c, induced by a transitive silencing signal produced by the primary target Y, is delayed when the upstream *gus* sequence in Y is separated by only 98 nt from the 3′ end region targeted by the X_{21}-derived primary siRNAs. The timing of silencing seemed to be negatively correlated with the distance between the directly targeted and adjacent sequences.

The Efficiency of *gus* Silencing Is Correlated with the Amount of Secondary siRNAs

The delay in *gus* silencing observed in some $X_{21}Y_*Z_c$ plants suggests that spreading of silencing along the Y_* transcripts proceeds gradually through the production of an increasing amount of secondary *gus*-specific siRNAs. Initially, amplification would result in a small amount of secondary siRNAs that is insufficient to induce strong suppression, but eventually enough *gus* siRNAs would be produced, leading to efficient transitive silencing of Z_c. To test this hypothesis, we evaluated the accumulation of siRNAs in nonsilenced Y_*Z_c plants as a control and in $X_{21}Y_*Z_c$ plants that were efficiently, intermediately, or not silenced. An RNA gel blot using a hydrolyzed ^{32}P-labeled probe comprising the full-length *gus*-coding sequence revealed *gus*-specific siRNAs in the low-M_r RNA fraction of $X_{21}Y_*Z_c$ plants that showed efficient (Fig. 3A, lanes 7–10) or intermediate silencing (lanes 5 and 6), whereas nonsilenced $X_{21}Y_{98}Z_c$ (lane 4) and Y_*Z_c plants (lanes 1–3) did not accumulate *gus* siRNAs. The signal intensities in intermediately silenced $X_{21}Y_*Z_c$ plants were clearly weaker than those in plants that had low GUS activities. Thus, there seems to be a correlation between the amount of secondary *gus* siRNAs produced and the reduction in GUS activity. Whether the intermediate levels are the result of overall intermediate siRNA accumulation or the sum of high levels of siRNAs in silenced and low levels in nonsilenced cells cannot be determined.

Increasing the Length of Sequence Homology between the Primary Target and the *CAT2* Endogene Increases the Frequency and Efficiency of Transitive *CAT2* Silencing

With the same constructs and plants, we checked whether the length of the *CAT2* inserts in the Y_* constructs was correlated with the frequency and efficiency of *CAT* silencing. The Arabidopsis *CAT2*



D

	No. of plants	5 weeks		6 weeks		8 weeks	
		gus-silenced plants	frequency	*gus*-silenced plants	frequency	*gus*-silenced plants	frequency
$X_{21}Y_7Z_c$	15	15	100%	15	100%	15	100%
$X_{21}Y_{98}Z_c$	15	12	80%	14	93%	15	100%
$X_{21}Y_{250}Z_c$	30	23	77%	25	83%	30	100%
$X_{21}Y_{500}Z_c$	15	10	67%	12	80%	15	100%

Figure 2. GUS activity in 15 $X_{21}Y_{98}Z_c$ (A), 30 $X_{21}Y_{250}Z_c$ (B), 15 $X_{21}Y_{500}Z_c$ (C), five Y_7Z_c (E), five $Y_{98}Z_c$ (F), five $Y_{250}Z_c$ (G), and five $Y_{500}Z_c$ (H) plants. The GUS activity was measured in protein extracts made of leaf tissue harvested at 5 weeks (diamond), 6 weeks (square), and 8 weeks (triangle). The GUS activity of the individual plants is represented as the percentage of the average GUS activity value of the corresponding Y_*Z_c plants and ordered according to decreasing GUS activities at 5 weeks. In D, the number of plants analyzed, the number of silenced $X_{21}Y_*Z_c$ plants (with a GUS activity value less than 10% of the average GUS activity of the corresponding Y_*Z_c plants), and the frequency of *gus* silencing are presented.

gene targeted by the Y_{98}, Y_{250}, and Y_{500} constructs is part of a gene family (*CAT1*, *CAT2*, and *CAT3*), with a nucleotide and amino acid sequence identity of 70% to 72% and 75% to 84%, respectively (Frugoli et al., 1996). The *CAT2* and *CAT3* genes are highly expressed in leaves. With the CAT activity assay (see ''Materials and Methods''), the total CAT activity was determined in protein extracts from leaves of 4-, 7-, and 8-week-old Y_*Z_c and $X_{21}Y_*Z_c$ plants (Fig. 4). The total CAT activity of the different transgenic plants is relative to the average CAT activity of the corresponding Y_*Z_c plants (100%). All Y_*Z_c plants (five plants for every Y_* construct) showed variable CAT activities, ranging from 40% to 159%, but this variation was comparable to that observed in wild-type plants (Fig. 4, E–H), so we can conclude that none of the Y_* constructs induces in cis- or in trans-silencing of *CAT* in the absence of locus X_{21}. Then we determined the total CAT activity in $X_{21}Y_*Z_c$ plants. The criterion for discriminating silenced from nonsilenced $X_{21}Y_*Z_c$ plants was a CAT activity value that was less than 10% of the average CAT activity of the corresponding Y_*Z_c plants. Nine out of 10 4-week-old $X_{21}Y_{500}Z_c$ plants (Fig. 4D) exhibited a delay in the onset of *CAT* silencing, with CAT activities ranging from 10% to 102% of the average CAT activity of the $Y_{500}Z_c$ plants (Fig. 4C). After 7 weeks, three out of 10 $X_{21}Y_{500}Z_c$ plants were not silenced based on this same criterion, but those plants had CAT activities of 12% to 18% and were silenced after 8 weeks. In contrast, none of the 20 $X_{21}Y_{250}Z_c$ plants showed silencing after 4 weeks (Fig. 4D), with CAT activities ranging from 11% to 160% (Fig. 4B). The percentage of silenced plants

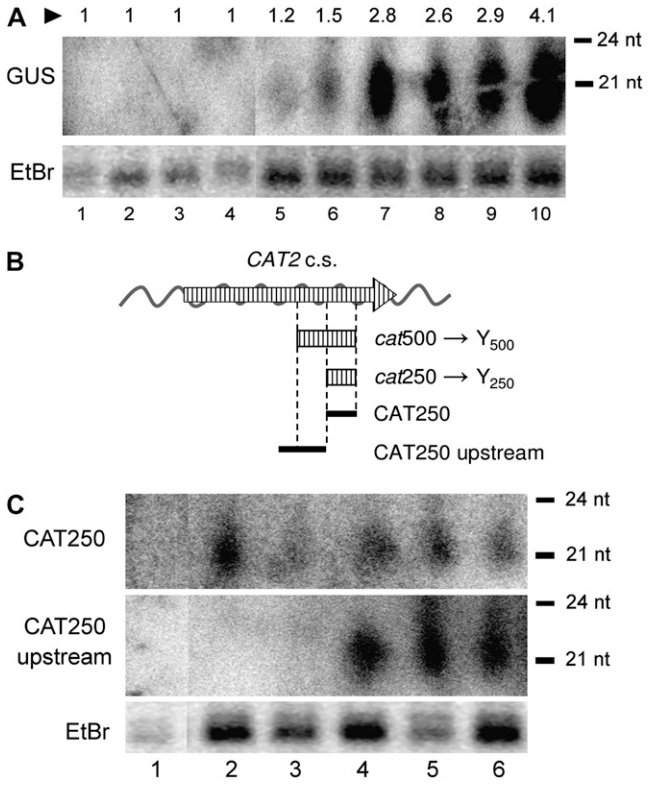


Figure 3. Accumulation of siRNAs. RNA oligomers of 21 nt and 24 nt were used as molecular markers. The predominant ethidium bromide-stained species in the low-M_r RNA fraction are shown as loading controls. The relative values of *gus* siRNAs for every $X_{21}Y_*Z_c$ sample are indicated above the lanes with the arrowhead. A, Detection of *gus* siRNAs. Each numbered lane contains the low-M_r RNA fraction extracted from leaves of individual plants at different time points. siRNAs were detected with a hydrolyzed ^{32}P-labeled probe comprising the full-length *gus*-coding sequence. Lane 1, $Y_{98}Z_c$; lane 2, $Y_{250}Z_c$; lane 3, $Y_{500}Z_c$; lane 4, $X_{21}Y_{98}Z_c$ not silenced for *gus* (98%); lane 5, $X_{21}Y_{250}Z_c$ with intermediate GUS activity (36%); lane 6, $X_{21}Y_{500}Z_c$ with intermediate GUS activity (36%); lane 7, *gus*-silenced $X_{21}Y_{98}Z_c$; lanes 8 and 9, *gus*-silenced $X_{21}Y_{250}Z_c$; lane 10, *gus*-silenced $X_{21}Y_{500}Z_c$. B, Schematic illustration of the position of the probes used to detect *CAT2* siRNAs (drawn to scale). The probe CAT250 corresponds to the *CAT2* insert (*cat*250) present in Y_{250}; the probe CAT250 upstream covers 398 nt of the *CAT2* sequence located upstream of *cat*250 and partially present in Y_{500} (*cat*500). C, Detection of *CAT2* siRNAs. Each numbered lane contains the low-M_r RNA fraction extracted from leaves of individual 7-week-old plants. Top, siRNAs detected with the CAT250 probe; bottom, siRNAs detected with CAT250 upstream. Lane 1, $Y_{250}Z_c$; lanes 2 and 3, *CAT*-silenced $X_{21}Y_{250}Z_c$; lanes 4 to 6, *CAT*-silenced $X_{21}Y_{500}Z_c$.

increased to 15% after 7 weeks, but after 8 weeks only 35% strongly suppressed CAT activities, although all the other plants had intermediate CAT activities, ranging from 10% to 37% of the average CAT activity of the $Y_{250}Z_c$ plants. None of the 14 $X_{21}Y_{98}Z_c$ plants (Fig. 4D) showed a strong reduction in total CAT activity after 4 and 7 weeks, with the CAT activities ranging from 50% to 165% and from 37% to 111%, respectively (Fig. 4A). After 8 weeks, still none of the $X_{21}Y_{98}Z_c$ plants were silenced based on the arbitrarily chosen silencing criterion, but two plants had a CAT activity of 10% and 18%, while the other 13 plants showed a total CAT activity of 52% to 116% (Fig. 4A). We conclude that only with primary target Y_{500} could a silencing frequency of 100% be obtained after 8 weeks, whereas with Y_{250} and Y_{98} not all $X_{21}Y_*Z_c$ plants reached strong *CAT* suppression. At this time point, the frequency and efficiency of silencing in $X_{21}Y_{250}Z_c$ plants were higher compared to those of $X_{21}Y_{98}Z_c$ plants. Thus, the extent of homology between the primary target and the endogene significantly influenced the degree of silencing and the probability of obtaining strong suppression.

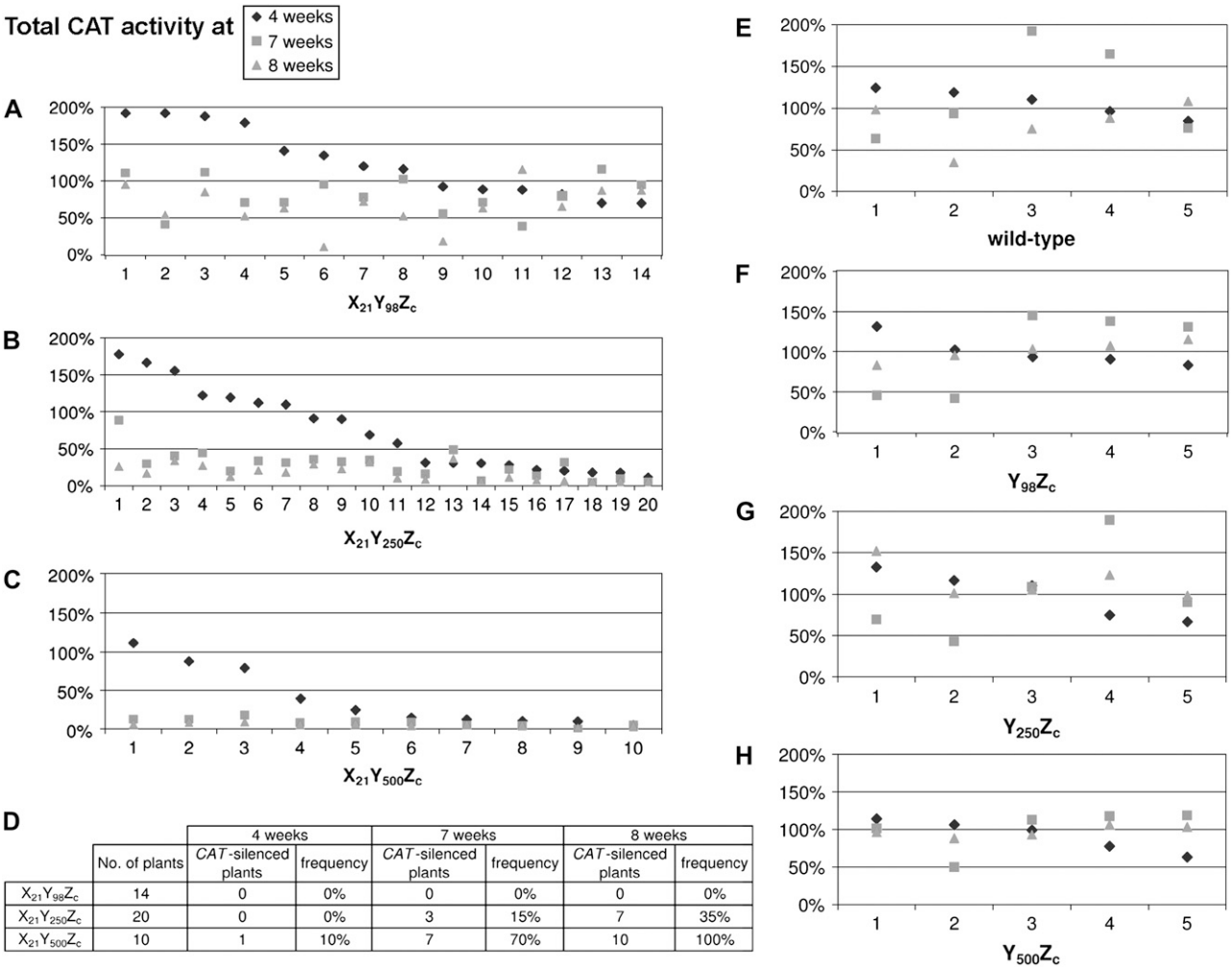
We also analyzed the *CAT2* RNA levels via quantitative real-time PCR in $X_{21}Y_{250}Z_c$ and $X_{21}Y_{500}Z_c$ plants that showed a very low CAT activity (Supplemental Fig. S2; Supplemental Protocol S1). RNA was extracted from leaf tissue, first-strand cDNA was generated with an oligo(dT)$_{12-18}$ primer, and a quantitative PCR was performed with *CAT2*-specific primers. Compared to the wild-type plant, a 15- to 50-fold reduction in *CAT2* RNA levels was measured in the $X_{21}Y_{250}Z_c$ and $X_{21}Y_{500}Z_c$ plants, confirming the silencing observed at the protein level.

A Transitively Silenced *CAT* Gene Does Not Give Rise to Detectable Tertiary siRNAs

To assess whether endogenous transcripts that are targeted by secondary siRNAs originating from primary target Y in turn produce tertiary siRNAs, we characterized the siRNA population in a nonsilenced $Y_{250}Z_c$ plant and in $X_{21}Y_{250}Z_c$ and $X_{21}Y_{500}Z_c$ plants that showed efficient *CAT* silencing. First, we used the hydrolyzed ^{32}P-labeled probe CAT250 corresponding to the *CAT2* insert present in Y_{250} (Fig. 3B), revealing that all $X_{21}Y_{250}Z_c$ and $X_{21}Y_{500}Z_c$ plants produced secondary *CAT2*-specific siRNAs (Fig. 3C, top, lanes 2–6), whereas in the $Y_{250}Z_c$ plant no siRNAs were detected (lane 1). Because it is not possible to discriminate between, on the one hand, secondary siRNAs originating from primary target Y and, on the other hand, tertiary siRNAs produced by endogenous CAT transcripts with this probe, we stripped the membrane and rehybridized it with the hydrolyzed riboprobe CAT250 upstream (Fig. 3B). With this probe we could detect secondary siRNAs originating from Y_{500} in $X_{21}Y_{500}Z_c$ plants (Fig. 3C, bottom, lanes 4–6), whereas in the $X_{21}Y_{250}Z_c$ plants no signal was detectable (Fig. 3C, bottom, lanes 2 and 3). This observation implies that endogenous CAT transcripts do not participate in the amplification of tertiary siRNAs, or that the endogenous mRNA-derived siRNAs were produced below detection level.

DISCUSSION

Here, we study in more detail the 3′ to 5′ spreading previously observed with the XYZ-silencing system (Van Houdt et al., 2003; Bleys et al., 2006a) by using primary targets Y_{98}, Y_{250}, and Y_{500} in which 98 nt, 250 nt, and 500 nt of the *CAT2* sequence, respectively, were



	No. of plants	4 weeks		7 weeks		8 weeks	
		CAT-silenced plants	frequency	*CAT*-silenced plants	frequency	*CAT*-silenced plants	frequency
$X_{21}Y_{98}Z_c$	14	0	0%	0	0%	0	0%
$X_{21}Y_{250}Z_c$	20	0	0%	3	15%	7	35%
$X_{21}Y_{500}Z_c$	10	1	10%	7	70%	10	100%

Figure 4. Total CAT activity measured in $X_{21}Y_{98}Z_c$ (A), $X_{21}Y_{250}Z_c$ (B), $X_{21}Y_{500}Z_c$ (C), wild-type (E), $Y_{98}Z_c$ (F), $Y_{250}Z_c$ (G), and $Y_{500}Z_c$ (H) plants, measured in protein extracts made of leaf tissue harvested at 4 weeks (diamond), 7 weeks (square), and 8 weeks (triangle). The total CAT activity of the individual plants is represented as the percentage of the average CAT activity value of the corresponding Y_*Z_c plants and ordered according to decreasing CAT activities at 5 weeks. In D, the number of plants analyzed, the number of *CAT*-silenced $X_{21}Y_*Z_c$ plants (with a CAT activity value less than 10% of the average CAT activity of the corresponding YZ_c plants), and the frequency of *CAT* silencing are presented.

inserted between the *nptII*-3′*chs* end targeted by the silencing inducer X_{21} and the upstream region homologous to a transgenic *gus* target Z_c. We demonstrate that the length of the *CAT2* inserts influences the timing and efficiency of silencing induced by secondary *CAT2*- and *gus*-specific siRNAs originating from the primary target transcripts. Increasing the distance between primary and secondary target regions in Y by larger *CAT2* inserts delays the onset of transitive *gus* silencing, whereas increasing the length of sequence homology with the endogenous *CAT2* target increases the frequency and efficiency of transitive *CAT* silencing.

According to the current model for transitive silencing in Arabidopsis, transcripts that are targeted by trigger-derived siRNAs can in some way be recognized by RDR6 as templates for the production of dsRNA (Vaistij et al., 2002; Himber et al., 2003; for review, see Bleys et al., 2006b). These new dsRNAs are subsequently processed into secondary siRNAs that can target homologous transcripts without sequence identity to the initial silencing inducer. Our findings suggest that transitivity requires time to build up a certain steady-state level of secondary siRNAs that results in a corresponding maximum plateau level of silencing. An increasing ratio of stochastically silenced to nonsilenced cells could account for the observed transition from no or less strong transitive silencing to an efficient silencing response through time. However, because transitive silencing is not a cell-autonomous process (Himber et al., 2003; García-Pérez et al., 2004), it is likely that a general secondary siRNA level is produced that can induce some degree of suppression.

Assuming that based on a certain amount of templates a corresponding number of secondary siRNAs can be made, increasing the length of substrate would result in a larger population of siRNAs and a

concomitantly increased degradation of the secondary target. Indeed, we observed that the length of sequence homology between the primary target and the endogene strongly influences the silencing response. With a length of 500 nt, efficient silencing can eventually be established in all plants, whereas a length of 250-nt homology results in less frequent and less efficient suppression, and a length of 98-nt homology has nearly no effect. Another factor that probably contributes to the efficiency of transitive silencing is the key enzyme RDR6. Consistently, both the activation and maintenance of posttranscriptional gene silencing or quelling in the fungus *Neurospora crassa* appear to rely on the amount of both the RDR QDE-1 and its transgenic RNA substrates (Forrest et al., 2004). Overexpression of *QDE*-1 resulted in a dramatic increase in the frequency of silencing, coinciding with an increase in the quantity of siRNAs. Accordingly, we observed a positive correlation between the amount of secondary siRNAs and the level of suppression.

Because primary X_{21}-derived siRNAs are targeted against the 3′ end of the Y_* transcripts, the secondary siRNAs are most probably produced from the region immediately upstream of this 3′ region. Consistently, some $X_{21}Y_{500}Z_c$ plants already show efficient suppression of *CAT* at 4 weeks, while they still have intermediate GUS activities at 5 weeks. However, after 8 weeks, all $X_{21}Y_*Z_c$ plants exhibit efficient *gus* silencing. This observation suggests that RDR6 needs time to reach the *gus* sequences in the primary targets and to produce a sufficient amount of *gus* siRNAs. However, because an endogenous sequence was used as insert into Y_* instead of a heterologous sequence without a target, this time dependency might be modulated by the involvement of the secondary *CAT2* siRNAs in the degradation of the endogenous *CAT2* target RNAs, which could interfere with the transitivity process along Y_* RNAs. Also, the secondary *CAT2* siRNAs could target Y-derived RNAs for degradation, resulting in lower accumulation levels of candidate template RNAs for RDR6. Nonetheless, our results indicate that RDR6 can overcome a length of at least 500 nt to give rise to *gus* siRNAs targeting Z_c. This observation is consistent with previous reports in *Nicotiana benthamiana* that demonstrated that virus-induced gene silencing can spread in a 3′ to 5′ direction at least through 332 nt (Vaistij et al., 2002; Petersen and Albrechtsen, 2005). When transgenic *gus*-expressing plants were inoculated with *Potato virus X* (PVX) carrying the 3′ fragment of the *gus*-coding sequence (termed S), siRNAs originating from the middle part of *gus* (termed U) were detected, whereas silencing induced with PVX-U did not give rise to siRNAs corresponding to the 5′ part of *gus* (termed G; Petersen and Albrechtsen, 2005). Therefore, it was concluded that 3′ to 5′ spreading does occur, but only for a limited distance of 600 nt (from S to U). However, we believe that 3′ to 5′ spreading over longer distances cannot be excluded because detection of secondary siRNAs was performed 1 week after PVX-U inoculation, thus leaving the possibility that, after a longer period, siRNAs could have spread further along the *gus* mRNA into the G region. Moreover, the G region might not allow transitivity because it has been shown to be a weak target (English et al., 1996; Braunstein et al., 2002) and it does not give rise to primary siRNAs (Hutvagner et al., 2000).

Independently of the possible distance of 3′ to 5′ spreading, amplification initiated from the 3′ end of the primary targets Y_{98}, Y_{250}, and Y_{500} would imply that a larger population of *CAT2* siRNAs is produced than that of *gus* siRNAs. This proposition is in agreement with the decreasing abundance of secondary siRNAs in *C. elegans* in function of the distance from the primary target region (Sijen et al., 2001). However, although all $X_{21}Y_*Z_c$ plants exhibit efficient *gus* silencing after 8 weeks, only some $X_{21}Y_{250}Z_c$ and hardly any $X_{21}Y_{98}Z_c$ plants obtain a strong reduction in CAT activity. This observation of plants with efficient *gus* silencing but inefficient *CAT* suppression is at first sight rather surprising. Combined with a postulated higher population of *CAT2* siRNAs, this suggests that the threshold level of siRNAs that is sufficient for efficient targeting of *CAT2* transcripts is higher than that of *gus* siRNAs. Another possible explanation could be a differential abundance of the *CAT2* and *gus* target transcripts, as lower levels of *CAT2* transcripts would require higher levels of siRNAs for efficient targeting. It could also be that the required siRNA threshold levels are the same for both the *gus* transgene and the *CAT2* endogene, but that the secondary *gus* target Z_c participates in the amplification of tertiary *gus* siRNAs, while the endogenous *CAT2* transcripts were found not to be template for the production of secondary siRNAs. Although it cannot be excluded that some secondary endogenous *CAT2* siRNAs are formed, the quantity was too low for detection, unlike secondary siRNAs originating from the Y_{500} transcript. Many other studies also could not provide evidence for transitivity along endogenous sequences (Vaistij et al., 2002; Himber et al., 2003; Kościańska et al., 2005; Miki et al., 2005; Petersen and Albrechtsen, 2005), whereas Sanders et al. (2002) detected secondary siRNAs originating from the endogenous β-1,3-glucanase gene in tobacco protoplasts and observed silencing of glucanase tester sequences homologous to the *glb* gene. It is very intriguing and unclear why an endogene-derived transcript would not act as template for RDR6, while a transgene-derived transcript can. Indeed, we observed transitivity along the transgenic *CAT2* sequences, in contrast to the endogenous *CAT2* transcripts. Thus, endogenous sequences seem to be protected from transitivity not by inherent features of the sequences themselves, but rather the context in which they are expressed.

MATERIALS AND METHODS

Constructs

For the cloning of constructs Y_{98}, Y_{250}, and Y_{500}, three PCR fragments *cat*98, *cat*250, and *cat*500 were synthesized using the Arabidopsis (*Arabidopsis thaliana*)

CAT2 gene (At4g35090) as template with one forward primer 5′-CCGGTTAATTAACGGCTTGCCAGCTTCTGTCC-3′ in combination with three different reverse primers: 5′-CCGGTTAATTAAGCCCTATCCGACCCACGCAT-3′ for *cat*98, 5′-CCGGTTAATTAAATGCTGAGAAGTATCCAACT-3′ for *cat*250, and 5′-CCGGTTAATTAAAATGAGCAACTTGCTTTCTG-3′ for *cat*500. The PCR fragments were cut with *Pac*I and inserted by ligation into construct Y_7 (Fig. 1; P35S-*gus*-*nptII*-3′*chs*) containing the full-length *gus*-coding sequence, also cut with *Pac*I. The sense orientation of the *CAT2* inserts was checked via PCR with the forward primer and a primer specific for the *gus* sequence. In the text, we used an asterisk (Y_*) to indicate all four Y constructs (either Y_7, Y_{98}, Y_{250}, or Y_{500}). All the T-DNA vectors were brought into the *Agrobacterium tumefaciens* strain C58C1Rif^R (pMP90).

Plant Material and Crosses

The production of plants harboring the silencing-inducing locus X_{21} and locus Z_c has been described previously (Bleys et al., 2006a). Locus X_{21} is an IR transgene locus with two convergently transcribed neomycin phosphotransferase II (*nptII*) reporter genes (P35S-*nptII*-3′*chs*) that show posttranscriptional gene silencing. Single-copy locus Z_c contains a highly expressed *gus* transgene (P35S-*gus*-3′*nos*). Transgenic plants homozygous for the Z_c locus were transformed with the different Y constructs by using floral dip (Clough and Bent, 1998). Seeds of the dipped plants were harvested and sown on K1 medium supplemented with phosphinothricin (10 mg L^{-1}) for selection of Y_{98}, Y_{250}, and Y_{500}. Fluorometric GUS assay and DNA gel-blot analysis of the T-DNA integration pattern were performed to select three primary transformants with a high GUS activity and single-copy locus of Y_{98}, Y_{250}, or Y_{500}, excluding in cis- and/or in trans-silencing of the *gus* transgenes in Y and Z_c. Progeny plants of these primary transformants were crossed with either transgenic plants homozygous for the X_{21} locus or wild-type Arabidopsis (ecotype Columbia). The resulting hybrid seeds were grown on medium selective for the presence of Y_{98}, Y_{250}, or Y_{500}. After 3 weeks, they were transferred to soil and grown under a light regime of 16 h day/8 h night at 21°C. Seeds of self-fertilized hybrid plants were harvested and sown on medium selective for the presence of either all three loci X_{21}, Y_{98}, Y_{250} or Y_{500}, and Z_c, or for Y_{98}, Y_{250} or Y_{500}, and Z_c. Because locus X_{21} and locus Z_c are located on the same chromosome, the resulting progeny plants, referred to as $X_{21}YZ_c$, are hemizygous for X_{21} and Z_c and either homozygous or hemizygous for Y. The progeny YZ_c plants contain both loci in either homozygous or hemizygous conditions. The selected progeny plants were transferred to soil after 3 weeks and grown under a light regime of 16 h day/8 h night at 21°C. Transitive silencing in these plants was studied at different time points after sowing.

Fluorometric GUS Assay

Five, 6, and 8 weeks after sowing on selective medium, protein extracts were prepared and GUS activity was measured as described by Van Houdt et al. (2003).

CAT Activity Assay

Four, 7, and 8 weeks after sowing, protein extracts were prepared from two rosette leaves, frozen in liquid nitrogen, by grinding in 120 μL extraction buffer (60 mM Tris-HCl, pH 6.9, 1 mm phenylmethylsulfonyl fluoride, 10 mM dithiothreitol, 20% glycerol). The homogenate was centrifuged (15,000*g*) twice at 4°C for 10 min to remove insoluble material. The supernatant was used for spectrophotometric CAT analysis according to Clare et al. (1984) after determining the total amount of soluble protein with the Bio-Rad Protein Assay (Bradford, 1976) using bovine serum albumin as a standard.

sRNA Analysis

sRNAs were detected as described by Van Houdt et al. (2003) with minor changes. The enriched low-M_r RNA fraction (35 μg) was loaded on gel, together with RNA oligomers as size controls. To detect *gus* siRNAs, a restriction fragment comprising the full-length *gus*-coding sequence was used, whereas for the *CAT2*-specific siRNAs, two different PCR fragments were utilized: CAT250, corresponding to the *CAT2* insert present in Y_{250}, and CAT250 upstream, covering 398 nt of the *CAT2* sequence located upstream of this insert. ^{32}P-labeled probes were synthesized with the Rediprime II random prime labeling system (GE-Healthcare) with [α-^{32}P]dCTP. After removal of unincorporated [α-^{32}P]dCTPs with the Bio-Spin p30 column (Bio-Rad Laboratories), the probe was hydrolyzed into fragments of approximately 50 nt. Hybridization and washes were performed as described at 50°C. Labeled membranes were exposed to a PhosphorImager screen (GE-Healthcare). Predominantly, ethidium bromide-stained species of low-M_r RNA, separated by agarose gel electrophoresis (1 μg/lane), were used as loading controls. The relative amount of siRNAs was calculated with the results of pixel volume quantitation (IMAGEQUANT software; GE-Healthcare).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AY113854.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. GUS activity in hybrid $X_{21}Y_*Z_c$ and Y_*Z_c plants.

Supplemental Figure S2. *CAT2* mRNA abundancy.

Supplemental Protocol S1. Quantitative real-time PCR.

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