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Spontaneous short-range silencing of a GFP transgene in *Nicotiana benthamiana* is possibly mediated by small quantities of siRNA that do not trigger systemic silencing

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Summary

A green fluorescent protein (GFP) transgene under the control of the 35S cauliflower mosaic virus (CaMV) promoter was introduced by *Agrobacterium*-mediated transformation into *Nicotiana benthamiana* to generate fourteen transgenic lines. Homozygous lines that contained one or two copies of the transgene showed great variation of GFP expression under ultraviolet (UV) light, which allowed classification into three types of transgenic plants. Plants from more than half of the transgenic lines underwent systemic RNA silencing and produced short interfering RNA (siRNA) as young seedlings, while plants of the remaining lines developed, in a spontaneous manner, defined GFP-silenced zones on their leaves, mostly in the form of circular spots that expanded to about 4–7 mm in size. In some of the latter lines, the GFP-silenced spots remained stable, but no systemic silencing occurred. Here we characterize this phenomenon, which we term spontaneous short-range silencing (SSRS). Biochemical analysis of silenced spot tissue did not reveal detectable levels of siRNA. However, agro-infiltration with the suppressor proteins P19 of cymbidium ring spot virus (CymRSV), HC-Pro of tobacco etch virus (TEV), and crosses to a P19 transgenic line, nevertheless suggests that low concentrations of siRNA may have a functional role in the locally silenced zone. We propose that small alterations in the steady-state concentration of siRNAs and their cognate mRNA are decisive with regard to whether silencing remains local or spreads in a systemic manner.

Keywords: P19, post-transcriptional gene silencing, RNA silencing, silencing suppressors, systemic signal.

Introduction

RNA silencing is the sequence-specific suppression of gene expression through the involvement of RNA. Related processes are found in most eukaryotes across kingdoms from fungi to mammals, as well as in plants, where the phenomenon is relatively well studied. Understanding silencing processes is complex, as various RNA molecules participate. In particular, double-stranded RNAs are important; these, together with resulting short RNA molecules, modulate gene expression by different, but partly related mechanisms. These include specific RNA degradation and translation arrest, but also chromatin changes that influence transcriptional activities (for recent summaries, see Cerutti, 2003; Finnegan and Matzke, 2003; Kidner and Martienssen, 2003). The biological function of RNA silencing in plants influences the regulation of gene expression in developmental processes (Carrington and Ambros, 2003; Hake, 2003; Kidner and Martienssen, 2003; Reinhart *et al.*, 2002), the suppression of transposon activity (Rudenko *et al.*, 2003) and the control of plant viruses, especially plant RNA viruses (Voinnet, 2001). To overcome RNA-based defence strategies of the host, many plant viruses encode a specific protein that interferes with the silencing process at various levels. The most prominent of the suppressors include p25 of potexviruses (Voinnet *et al.*, 2000), the 2b protein of cucumoviruses (Brigneti *et al.*, 1998; Lucy *et al.*, 2000), HC-Pro of potyviruses (Anandalakshmi *et al.*, 1998; Brigneti *et al.*, 1998) and P19 of tombusviruses (Silhavy *et al.*, 2002; Vance and Vaucheret, 2001; Voinnet *et al.*, 1999), and a recent review

summarizes the specific functions of these suppressors (Silhavy and Burgyan, 2004).

RNA silencing has typically at least three distinct stages: initiation, maintenance and systemic spread (Vaucheret *et al.*, 2001). The key trigger is a double-stranded RNA that is processed into short interfering RNA (siRNA), which are the hallmark of RNA silencing (Hamilton and Baulcombe, 1999). In *Drosophila*, this reaction is catalysed by an RNase III-type enzyme named Dicer (Bernstein *et al.*, 2001). Related enzymes have been found in other organisms, but the number of Dicer enzymes varies. In *Arabidopsis thaliana*, four genes have been identified, and named (or re-named) Dicer-like 1 to 4 (*Dcl1–4*) (Schauer *et al.*, 2002), and recently their involvement in silencing pathways has been analysed (Gasciolli *et al.*, 2005; Xie *et al.*, 2004; Xie *et al.*, 2005). In accordance with current models, double-stranded RNA is a reliable inducer of the RNA silencing process. This accounts for the double-stranded RNA intermediates of plant viruses and even of viroids that either replicate in the nucleus (Denti *et al.*, 2004; Itaya *et al.*, 2001; Papaefthimiou *et al.*, 2001) or chloroplast (Martinez de Alba *et al.*, 2002), but also for engineered hairpin constructs, which result in the generation of siRNAs and confer reliable viral resistance (Kalantidis *et al.*, 2002; Missiou *et al.*, 2004; Smith *et al.*, 2000). In addition to direct induction by double-stranded RNA, single-stranded RNA, especially when transcribed from a transgene, is also able to induce RNA silencing. Details of this step are not clear, but it is believed that RNA-directed RNA polymerase (RdRP) plays a decisive role in the synthesis of double-stranded RNA from a single-stranded RNA precursor (Dalmay *et al.*, 2000). However, the conversion seems to be an inadvertent event, whose likeliness increases with the level of gene expression (threshold model), presumably because the single-stranded RNA exhibits some distinguished irregular features that make it a template for RdRP. Therefore it is called aberrant RNA, and this term also expresses the lack of understanding about the nature of this RNA species. Although RNA silencing is induced occasionally by single-stranded RNA and reliably by double-stranded RNA, its initiation is not always sufficient for the perpetuation of silencing (Palauqui and Vaucheret, 1998). It has been proposed that epigenetic changes (e.g. DNA and histone methylation) may be involved in the maintenance step of silencing but little is known about this step.

The next step in RNA silencing is its systemic spread throughout the plant in a non-cell-autonomous manner that is reminiscent of the systemic spread of viruses. By grafting experiments, Palauqui and Vaucheret (1998) and Voinnet *et al.* (1998) have unequivocally demonstrated that silencing can systemically spread from a silenced stock to a non-silenced scion. The exact make-up of the 'mobile signal' that is responsible for long-distance systemic spread or RNA silencing, however, remains to be determined (Baulcombe, 2002; Fagard and Vaucheret, 2000; Mlotshwa *et al.*, 2002). There is increasing evidence that a second mechanism exists that is responsible for short-range cell-to-cell spread of RNA silencing (Himber *et al.*, 2003; Ryabov *et al.*, 2004; Silhavy *et al.*, 2002). Using agro-infiltration of a green fluorescent protein (GFP) construct or movement-deficient recombinant viruses, RNA silencing could be induced in defined zones corresponding to about 13 cells (Himber *et al.*, 2003; Silhavy *et al.*, 2002). This short-range silencing was observed as the earliest evidence of silencing before it spread throughout the infiltrated zone and eventually systemically throughout the plant. In a broader sense, short-range silencing had been reported to occur before, either spontaneously (Palauqui *et al.*, 1996) or induced in tobacco and petunia (Klahre *et al.*, 2002; Palauqui and Balzergue, 1999; Que and Jorgensen, 1998; Que *et al.*, 1998), as the appearance of silenced non-clonal spots.

In this paper, we characterize GFP-expressing transgenic *Nicotiana benthamiana* lines that exhibit spontaneous short-range silencing (SSRS) without any specific external inductor. Silencing in our transgenic lines initiates either as GFP-silenced spots or silenced segments of the vascular tissue or both. When silenced segments appear along vascular tissue, the plant always progresses to systemic silencing. However, in some lines, silencing remains almost exclusively restricted to a circular spot. We provide evidence that this short-range spread moves primarily, but not solely, through plasmodesmata, and is likely to be the result of post-transcriptional gene silencing (PTGS) as opposed to transcriptional gene silencing (TGS). This short-range silencing is also likely to be related to siRNA signalling as it can be suppressed by the silencing suppressor P19 of cymbidium ring spot virus (CymRSV). We have managed to induce SSRS in an otherwise stably expressing GFP line by local expression of antisense GFP at concentrations inefficient to cause systemic silencing.

Results and discussion

GFP transgenic lines

It is well established that PTGS can be induced in plants efficiently by double-stranded RNAs. However, single-stranded RNA may also initiate the process, especially if the RNA originates from a transgene (reviewed by Vaucheret *et al.*, 1998). Once initiated, RNA silencing has the potential to spread systemically in the entire plant.

Using a GFP transgene, we have analysed the potential for spontaneous silencing. As previously described, we found that the same gene construct may induce different phenotypes. The differences found have been attributed to positional effects (Day *et al.*, 2000; van Leeuwen *et al.*, 2001), transgene complexity (Qin *et al.*, 2003) or transgene expression levels (Forsbach *et al.*, 2003; Lechtenberg *et al.*, 2003; Que *et al.*, 1997; Schubert *et al.*, 2004), most probably

depending on the site of chromosomal integration. Using a single vector construct, we generated 14 lines that were transgenic for *mGFP4* under the control of the cauliflower mosaic virus (CaMV) 35S promoter. All plants were selfed and the segregation rates of the T_1 seedlings on selection media were determined. Segregation patterns were followed up to T_4 seedlings. Based on this, together with Southern hybridization data for representative lines from each type (Figure S1), we concluded that these lines carried one or two copies of the transgene (data not shown). Homozygous plants were used for the experiments described below. We observed a great degree of variation in GFP expression amongst these lines, and Northern hybridization showed that the level of GFP mRNA corresponded roughly to the degree of fluorescence (Figure S2). With the exception of leaf material from line 4.4 (type III line, see below), all leaf material analysed in the above Northern blots was from non-silenced young leaves. In general, three major types (I–III) of GFP expression pattern could be discriminated (Figure S3).

Type I lines (4 of 14) showed the most intense levels of GFP expression, which was uniformly strong in all leaves and the stem. However, inspection under ultraviolet (UV) light revealed that some leaves developed, without any noticeable inductor and in a spontaneous manner, some well-defined red spots (due to the fluorescence of chlorophyll, Figure 1a) or short silenced segments along secondary or tertiary vascular tissue, suggesting that these zones were GFP-silenced. Soon after the appearance of such red zones, type I plants underwent systemic silencing for GFP. Almost every type I plant showed eventually systemic silencing, although it could not be predicted at what age the systemic silencing would initiate, indicating that a spontaneous event provided the starting signal, first for the initial red zones and subsequently for systemic silencing. Variability in the onset of silencing was observed both between different type I lines and also within individual transgenic lines. Exceptionally, plants reached maturity without developing red zones and without systemic silencing spreading through the plant. Silenced spots, however, could be detected in all plants.

Type II lines (2 of 14) showed much lower levels of GFP expression compared with type I plants, which resulted in an orange phenotype under UV light. Like type I plants, type II plants also spontaneously developed red spots during some stage of their development (Figure 1b). However, in most type II plants, the occurrence of red spots did not result in any systemic silencing, unlike in type I plants. Instead, more and more GFP-silenced spots appeared on their leaves. Of the more than 300 plants inspected so far, only about 5% eventually underwent systemic silencing, exclusively when plants were old.

Type III plants (8 of 14) showed full RNA silencing extremely early, at the seedling stage. We have previously shown that siRNAs can be detected in RNA preparations from a type III line (Boutla *et al.*, 2002), suggesting that RNA silencing is responsible for suppressing GFP expression. In the same study, we also showed that extracts prepared from type III plants were potent inducers of gene silencing, even across kingdoms.

Collectively our analysis showed that all our 14 GFP lines eventually underwent some RNA silencing, but the type of silencing varied depending on the category of plants. Sequencing of GFP cDNA from transgenic plants revealed no sequence deviations from the original (data not shown).

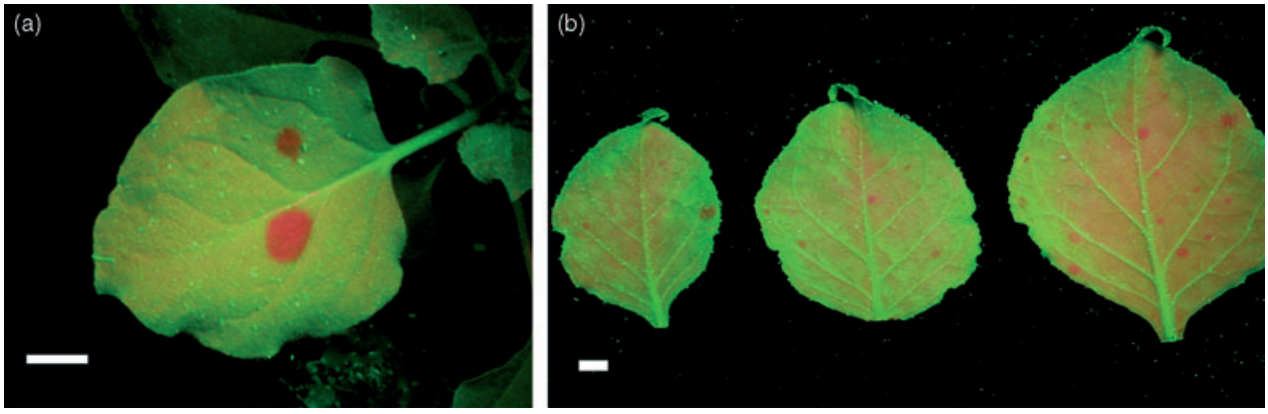

Figure 1. Morphology of spontaneous short-range silencing (SSRS).
(a) An SSRS spot on a leaf from a type I plant of line 12.1. The local suppression of GFP appears as intense red circular spots (due to auto-fluorescence of the chlorophyll) on both sides of the leaf, with diameters reaching about 5 mm, exceptionally 7 mm. Spots on type I plants are frequently followed by systemic silencing. Size marker, 1 cm.
(b) Three leaves from an individual 6-week-old, type II plant of line 5.3 originating from the upper, middle and lower part (left to right); all leaves show SSRS spots, which can be clearly discriminated, despite the relatively low GFP expression. Spots are more frequent on the older leaf and are generally more frequent than in type I plants. Size marker, 1 cm.

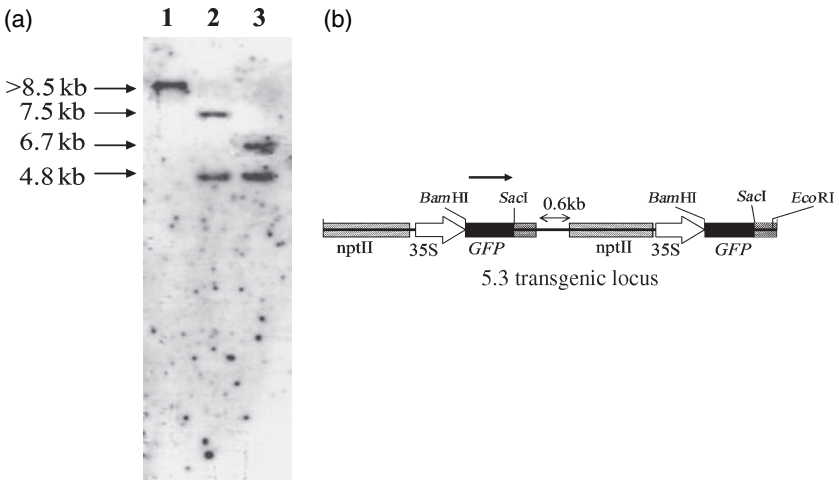

Figure 2. The GFP sequence in the transgenic region in type II transgenic line 5.3 is present as a direct repeat.
(a) Southern hybridization of genomic DNA from 5.3 line. Lanes 1, 2 and 3, *Eco*RI-, *Sac*I- and *Bam*HI-digested samples, respectively. The result was confirmed by hybridization with promoter sequences (not shown).
(b) Region containing the GFP transgenic sequences of type II GFP line 5.3. The transgene is integrated as a direct repeat, with the two copies of the GFP gene separated by approximately 2.0 kb. The *Bam*HI/*Sac*I-digested GFP sequence was used to generate a DNA probe (arrow).

In type I plants, SSRS was followed by systemic silencing, while SSRS was stable in type II plants. This indicates that spontaneous systemic silencing is the result of two consecutive processes controlled by separate mechanisms that can be dissected in the transgenic lines that we describe. The circular spots are also distinct from the streaks of silenced tissue along secondary and tertiary veins in type I plants, which always indicated the eventual onset of systemic silencing

It has been shown that *N. benthamiana*, the plant species used in our experiments, carries a specific variant of RdRP that is associated with increased susceptibility to viruses (Yang *et al.*, 2004). It is possible that this specificity of *N. benthamiana* may be related to the exceptionally high percentage of spontaneous silencing observed in our transgenic lines. Nevertheless, spontaneous silencing has been repeatedly reported in various other plant species that are likely to harbour unaltered variants of RdRPs (Crete *et al.*, 2001; Elmayan and Vaucheret, 1996; Qin *et al.*, 2003).

We were interested to analyse type II plants in greater detail, in which GFP silencing occurs regularly but is confined to certain well-defined spots that do not induce systemic silencing. We term this phenomenon SSRS. To standardize the experiments, we worked, unless otherwise noted, with type II GFP line 5.3. A more detailed Southern analysis of homozygous T4-generation line 5.3 plants (type II) revealed that line 5.3 carries a direct repeat of the GFP construct (Figure 2).

General properties of SSRS

SSRS normally appeared in plants of line 5.3 in source leaves, but occasionally spots could also be observed in sink leaves. The earliest occurrence was detectable in two-leaf seedlings. We have only observed SSRS on leaves, but they may be harder to detect in other tissues. Within leaves, we did not observe a bias towards some specific tissues. We observed SSRS in intervascular areas of the lamina, but also in areas that include the vascular tissues, including the primary vein of the leaf. The number of spots increased with time, and some mature leaves carried more than 10 spots (Figure 1b). The radius of individual spots grew from initially less than 2 mm to typically about 4–5 mm with a maximum of about 7 mm (Figure 1a). To test the influence of the ploidy level, we crossed a homozygous plant of line 5.3 with a wild-type plant to generate plants that were hemizygous for the GFP transgene of line 5.3. Here we saw a sharp decrease in the frequency of SSRS, while the size of the spots remained the same (not shown). While the frequency of SSRS was influenced by the genetic background, its actual appearance seemed to be an endogenous stochastic process. Under any of the growth conditions tested, either in the greenhouse or at two different light and temperature regimes in the growth chamber (see Experimental procedures) SSRS of GFP appeared at similar frequencies in 5.3 plants.

Microscopy analysis of the SSRS zone

Microscopy of early stages of SSRS detected some GFP expression in the entire emerging spot zone, including its centre. But GFP expression was generally lower compared to the non-silenced area (Figure 3a). Later, GFP became almost fully suppressed in the centre of the SSRS zone, including stomata (Figure 3b). The fully suppressed zone was surrounded by a more or less circular zone, where GFP could only be observed in stomata (Figure 3b–d). Finally, at the border of the silenced area, a zone of lower GFP expression could be observed (Figure 3a,d). Confocal microscopy revealed that, in a three-dimensional model, the silenced spots would appear as imperfect spheres (not shown).

These data and the radial shape of the spots suggest that the SSRS originates from a 'silencing centre' that induced silencing in the entire SSRS zone. The centre of the zone is distinct, as only in this zone are the stomata also GFP-silenced. The spreading of this short-range silencing signal

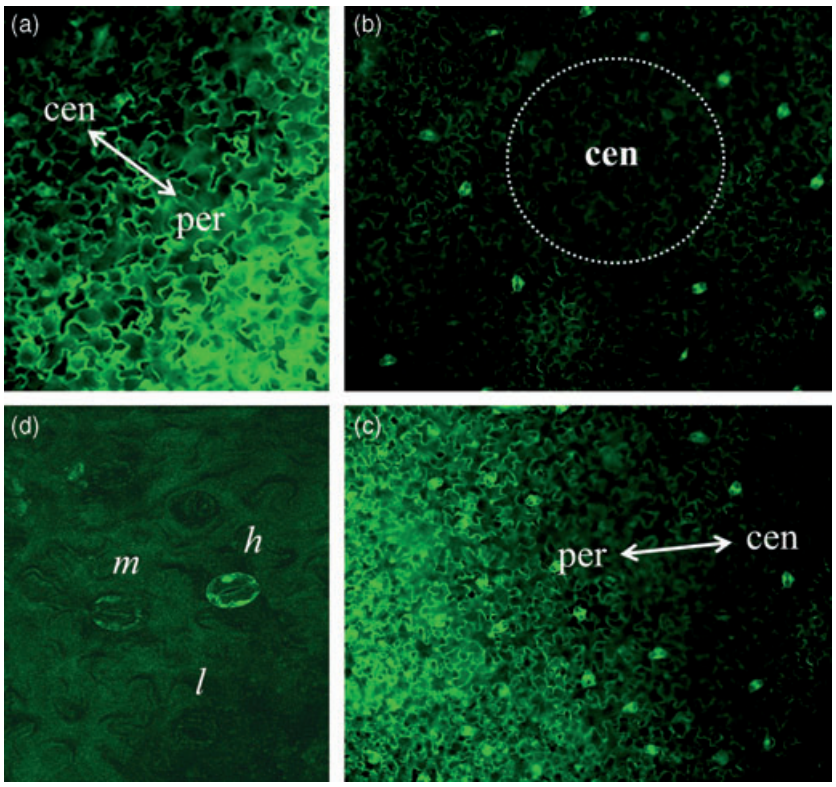

Figure 3. Microscopy of SSRS zones (spots) from a type II plant under ultraviolet light.
(a) Section across the border of an SSRS zone at an early stage. The centre (cen) and the periphery (per) of the spot are indicated.
(b) The centre of a mature silenced spot. The stomata in the centre do not express GFP, and are visible as green foci in the rest of the SSRS zone.
(c) Detail of an area immediately adjacent to the centre of the spot; guard cells with low (*l*), medium (*m*) and high (*h*) GFP fluorescence can be found.
(d) The periphery of the same spot as in (b). A gradual change in GFP fluorescence can be observed, but most guard cells still fully express GFP. The images were acquired using a scanning confocal microscope, filtering out chlorophyll fluorescence.

seems to be primarily through plasmodesmata, as stomata, which are symplastically isolated at this stage, appear to be silenced last. Eventually, stomata in the core of the SSRS zone are also silenced, indicating that they receive the signal via a slower apoplastic route.

The final size of the silenced spots varied, but the majority of spots were about 4–5 mm in diameter, which corresponds to about 25–35 epidermal cells when they reach full size. The silenced area appears to always have the same size on both sides of the leaf. This size range is in good agreement with recent agro-infiltration experiments with a GFP gene in GFP-expressing transgenic plants, which resulted in a red (silenced) border around the strongly fluorescent infiltrated area (Himber *et al.*, 2003; Silhavy *et al.*, 2002). This red zone was attributed to limited cell-to-cell movement of a signal triggered in a small number of cells (Himber *et al.*, 2003). The size of this zone has been estimated as 13 ± 2 cells. Although silencing in this system was triggered by agro-infiltration and always resulted eventually in systemic silencing, it shares commonalities with SSRS as the local short-range silencing precedes the systemic silencing. Our type II plants show that local silencing may be uncoupled from the ensuing step that results in systemic silencing.

The phenomenon of local silencing followed by systemic silencing has been observed previously by Palauqui *et al.* (1996), who introduced an additional copy of nitrite reductase to tobacco. This lead to the stochastic appearance of small chlorotic areas on the leaves that soon spread systemically to the rest of the plant. This is equivalent to our type I plants, but type II plants have to our knowledge not been described.

The spots we describe here as a result of SSRS are reminiscent of the spots detectable after bombardment of leaves with either DNA (Palauqui and Balzergue, 1999) or siRNA (Klahre *et al.*, 2002) and the silenced foci induced by a viral vector (Ryabov *et al.*, 2004). In our transgenic lines, however, there is no external trigger. Silencing that only spreads conditionally has been described in the worm *Caenorhabditis elegans* (Timmons *et al.*, 2003), although there may be significant differences in the mechanism of systemic silencing between nematodes and plants.

Analysis for siRNAs in SSRS zones

It was likely that the centre of the SSRS spot generated double-stranded GFP RNA and thus siRNAs that would spread out to the surrounding zone.

Therefore, we wished to analyse for GFP-specific siRNAs in SSRS zones. This is not a trivial task in view of the limited amount of tissue. For that reason, we collected the material from more than 50 spots (corresponding to about 200 mg of fresh tissue), which was collectively extracted and analysed. If a comparable amount of leaf material originating from truly silenced tissue is analysed, siRNAs are easy to detect (Figure 4a). However, we were unable to detect GFP-specific siRNA in the collected spot zones. Instead, we could detect a

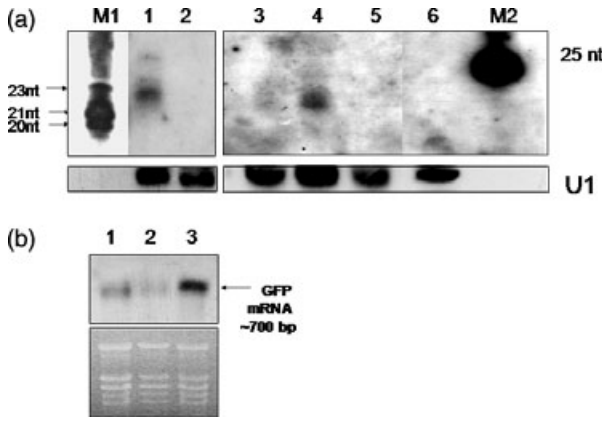

Figure 4. Northern analysis for the detection of GFP siRNAs and mRNA.
(a) siRNA analysis. Lane 1, tissue actively undergoing systemic silencing; lane 2, fully green-fluorescent tissue from a non-silenced plant; lane 3, leaf tissue showing initiation of silencing along the veins; lane 4, leaf tissue from a type I plant with mixed silenced and non-silenced areas; lane 5, tissue from collected SSRS areas; line 6, green tissue from a leaf showing spots; M1 and M2, size markers with the nucleotide numbers indicated. The lower part shows a control hybridization of the same membrane detecting U1 RNA to ensure equal loading.
(b) mRNA analysis. Lane 1, RNA from a type I leaf undergoing silencing for GFP, with silenced and non-silenced tissues; lane 2, RNA extracted from SSRS regions; lane 3, RNA from non-silenced type II line tissue. Bottom part: ethidium bromide staining of the gel to ensure equal loading of the samples.

low concentration of GFP mRNA (Figure 4b). As the excision of spots under UV light is technically difficult, we cannot completely rule out the possibility that some GFP-expressing tissue was transferred to the 'spot fraction'. However, a moderate expression of GFP is also in agreement with what can be seen microscopically. The lack of detectable quantities of siRNAs indicates that most cells of the SSRS zone are unable to produce siRNAs in significant quantities, suggesting that only part of the silencing pathways had been activated or that only a few cells in the centre of the SSRS were generating siRNA, without, however, producing the signal necessary for systemic spread.

Silencing suppressors P19 and HC-Pro but not an additional GFP copy can suppress SSRS

In order to characterize the SSRS process, we followed two additional approaches. First we tested whether GFP expression can be achieved in the silenced foci by ectopic expression of GFP. Leaves with SSRS were agro-infiltrated with bacteria carrying GFP-expressing constructs. Local over-expression of GFP was soon observed in the agro-infiltrated area but not in the SSRS zones (Figure 5a). It is therefore highly unlikely that the silenced spots in this case are the result of TGS as it is improbable that all the additional GFP copies infiltrated were silenced at the transcriptional level. In accordance with this, analysis of the 35S promoter sequence of 5.3 line with an appropriate restriction enzyme (*Hae*III) did not reveal methylation in the promoter region (not shown). Methylation of the promoter has been previously shown to result in TGS (Mette *et al.*, 2000; Park *et al.*, 1996). Nevertheless, it is plausible that this direct repeat may have unusual methylation patterns, or that the open reading frame, as a result of the specific integration pattern, may be refractory to methylation to propagate silencing.

We then crossed the GFP 5.3 line with the stably GFP-expressing 16c line (5.3 × 16c and 16c × 5.3). All F_1 plants were GFP fluorescent and showed SSRS, although at a lower frequency than the homozygous 5.3 plants (on average 6–8 spots per plant compared to more than 20 in the homozygous 5.3 plants) (not shown). The GFP copy of the 5.3 line therefore seems to destabilize GFP expression even in the hemizygous state.

Next we agro-infiltrated two viral suppressors of silencing, each under the control of the CaMV 35S promoter, in the area adjacent to SSRS. Local application of P19 of CymRSV re-established GFP expression in the SSRS tissue (Figure 5c,d). P19 has been unequivocally shown to suppress PTGS by specifically binding siRNAs (Lakatos *et al.*, 2004; Silhavy *et al.*, 2002; Vargason *et al.*, 2003). CymRSV suppression of silencing could first be observed as a mild increase of green fluorescence in the silenced tissue at day 5 post-infiltration (not shown) and GFP fluorescence was fully recovered at day 12 post-infiltration (Figure 5d). These results were reproduced with leaves of different ages and with four plants of GFP line 5.3. A similar pattern of GFP recovery was also observed for HC-Pro of tobacco etch virus (TEV) (Figure 5e,f). In both cases, recovery of GFP expression as observed by fluorescence was not always complete, possibly because of incomplete removal of siRNAs and/or time constraints of the agro-infiltration technique. We then crossed line 5.3 to a line stably expressing the silencing suppressor P19 of artichoke mottle crinkle virus (AMCV) (Silhavy *et al.*, 2002). F_1 progeny showed no SSRS, but observation is difficult due to the low GFP expression of heterozygous 5.3 plants (not shown). In the F_2, all plants homozygous for GFP 5.3 carrying at least one copy of P19 were free of SSRS (Figure 5b). Exceptionally, one or two spots could be observed in few double homozygotes (3 of 16 of the P19 × 5.3 cross). This could be due to incomplete removal of siRNAs in the P19-expressing lines.

Suppression of SSRS in the presence of P19, which acts as a molecular caliper to specifically bind *bona fide* siRNAs based on the length of the duplex region of the RNA (Lakatos *et al.*, 2004; Vargason *et al.*, 2003; reviewed by Baulcombe and Molnar, 2004), suggests that a low concentration of siRNAs is involved in the formation of SSRS. It is not clear how exactly HC-Pro suppresses silencing (Mallory *et al.*, 2001; Mallory *et al.*, 2002; Mette *et al.*, 2001). However, there is recent evidence (J. Burgyon, L. Lakatos, Agricultural Biotechnology Center, Gödöllő, Hungary, pers. comm.) that

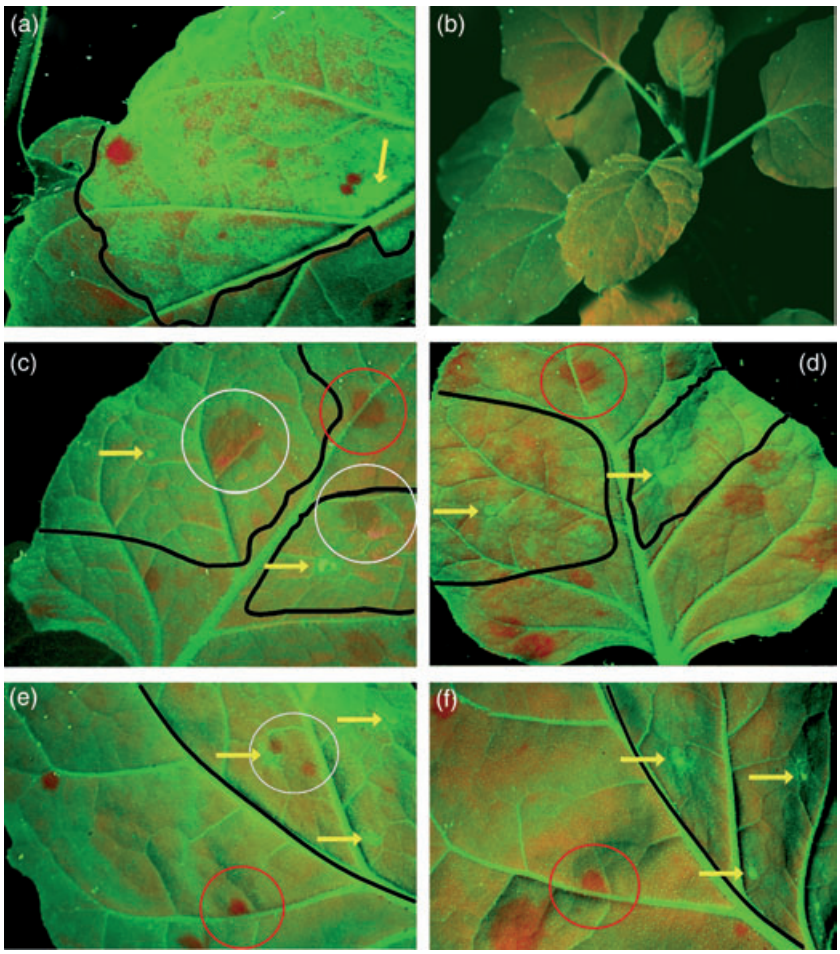

Figure 5. P19 and HC-Pro but not additional GFP copies can suppress SSRS.
(a) Local over-expression of GFP, 4 days after agro-infiltration; additional expression of GFP cannot overcome silencing in the SSRS zone.
(b) An F_2 plant from an A30 × 5.3 cross, homozygous for GFP and carrying at least one copy of P19 is free of spots.
(c,d,e,f) Expression of the silencing suppressors P19 of CymRSV and HC-Pro of tobacco etch virus; the white-encircled spots are in the infiltrated zone- the red-encircled spot is outside the infiltrated zone. The same leaf is shown (c) 1 and (d) 12 days post-infiltration. GFP expression is re-established in the SSRS zones agro-infiltrated with P19 (c, 1 day and d, 12 days post-infiltration) and HC-Pro (e, 1 day and f, 12 days post-agro-infiltration); spots outside remain unaffected. Yellow arrows mark the sites of injection and black lines the border of the infiltrated zone.

HC-Pro also binds siRNAs, and it is therefore not surprising that it also manages to suppress SSRS. Nevertheless, it is possible that HC-Pro interferes with silencing in the silenced spots in more than one way.

On the basis of the above findings, we propose that the SSRS zone contains a low level of siRNAs (below detection level) compared with normal tissue that has undergone systemic silencing. This notion is also in agreement with the observation by Klahre *et al.* (2002) that direct delivery of siRNA by biolistic methods can induce silenced zones, but will not induce systemic silencing. Initially, siRNAs need to be generated, most likely from a double-stranded RNA precursor. The siRNAs are sufficient to direct the degradation of GFP mRNA and to induce SSRS. The circular shape and acropetal growth of a SSRS zone suggests an initial source in the centre of a spot consisting of very few cells, perhaps even a single cell, generating a short-range silencing signal, most likely siRNAs. The exact position and time of occurrence is a stochastic and unpredictable process for which no inducer could be identified so far. However, each transgenic line has its own intrinsic likeliness of forming such core silencing units.

Excision of silenced spot areas and grafting experiments

The agro-infiltration experiments with P19 suggested that the SSRS zone contained functional GFP siRNAs. It is postulated that the centre of the SSRS contains a source that constantly produces double-stranded GFP RNA that is converted to siRNAs, which then diffuse out into the periphery to direct mRNA degradation. The concentration of siRNAs may be low, but is sufficient to direct degradation of the GFP mRNA. In order to test whether the silencing centre is necessary for the growth or even maintenance of silencing in the spot, we mechanically excised the silenced spots using a Pasteur pipette (outer diameter 1.6 mm). When only part of the silenced spot was removed this way, either from the centre or the periphery, GFP expression did not recover in any of the silenced tissue and sometimes the silenced area even continued to grow. In contrast, when all silenced tissue was removed no silencing appeared in adjacent tissue (Figure 6). Therefore, it seems likely that once a cell has been converted to a silenced one it can maintain silencing irrespective of whether further silencing signals flow in.

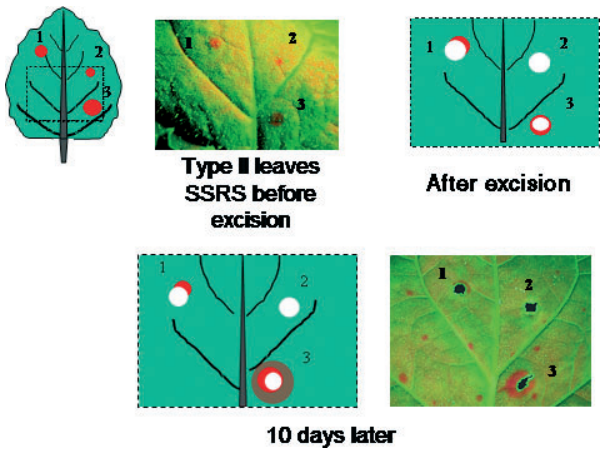

Figure 6. Spot excision experiment.
We selected a leaf from a type II plant that contained three SSRS spots of different size. Using a Pasteur pipette (outer diameter 1.6 mm), we cut through the centre of spot 1, while spot 2 was punched out and spot 3, which was older and larger, had its centre removed. After 10 days, spots that had all of the silenced area removed showed no spread (spot 2), whereas spots that had their centre or periphery removed did not re-establish GFP expression. Note that new small spots have arisen in the leaf.

The GFP agro-infiltration experiments had shown that type II plants are able to undergo systemic silencing if a regular silencing signal is provided (Figure S4). However, there was the possibility that type II plants require a higher dose of the silencing signal. To test this possibility we conducted the following grafting experiments. First, we grafted the stably GFP-expressing line 16c that is sensitive to RNA silencing onto type II plants and type I plants as a control. Scions of 16c plants grafted onto type I stocks showed, as expected, spreading of silencing at14 days post-grafting (Figure S5a). However, line 16c scions grafted onto type II plants showed no silencing at any time point (Figure S5b), suggesting that type II plants do not produce a signal for systemic silencing or for spread of SSRS. In contrast, grafting of type II scions onto type I stock was as efficient in silencing as grafting of 16c scions (Figure S5c).

Our experimental data strongly suggest that the lack of systemic spread of silencing in type II plants is due to an insufficient production of the silencing signal rather than a decreased sensitivity for the silencing response.

SSRS can be induced by local expression of antisense RNA

Agro-infiltration of line16c leaves with constructs transcribing dsGFP under a 35S promoter induced local silencing in the agro-infiltrated area a few days post-infiltration, and is effective even at low bacteria concentrations. The same effect may be achieved by agro-infiltration of line 16c leaves with agrobacteria transcribing *35S*-driven antisense GFP RNA, although less efficiently than with the ds-producing construct (not shown). However, when we infiltrated 16c leaves with agrobacteria carrying the antisense GFP construct as above but at low inoculum concentration (optical density 0.1 which here corresponds to approximately 10^7 bacteria per ml), we initially noticed no effect on the agro-infiltrated area. At such low concentrations, we found that the agro-infiltrated area did not necrotize and continued to develop normally. Eventually, 4–6 weeks post-infiltration all six leaves agro-infiltrated with the antisense GFP construct at low bacterial concentration developed silenced spots in the agro-infiltrated area (Figure 7a). Control agro-infiltration with agrobacteria carrying no GFP-related plasmid (Figure 7b) had no effect on GFP expression. Agro-infiltration of bacteria carrying a sense GFP-transcribing construct at similarly low concentrations as above usually did not result in the induction of silenced spots. Nevertheless, spot induction was exceptionally observed following sense GFP over-expression (Figure S6).

At present, no conclusive answer can be given as to what the actual inducer of systemic silencing is. However, it is possible that the steady-state concentration of siRNAs plays an important role. We propose that a series of thresholds may control the onset of short-range and then of long-range/systemic silencing in plants. According to our model, when a silencing trigger, possibly some aberrant RNA, is 'sensed' in a cell, the quantity of the trigger may be crucial in determining if and how silencing is going to proceed. If it does not exceed a certain threshold (and therefore may not constitute real danger), it may be ignored altogether. If it exceeds a hypothetical threshold, it would cause immediate cellular silencing that would then spread a short distance (short-range silencing). If the trigger exceeds a second hypothetical threshold, the next level of silencing that

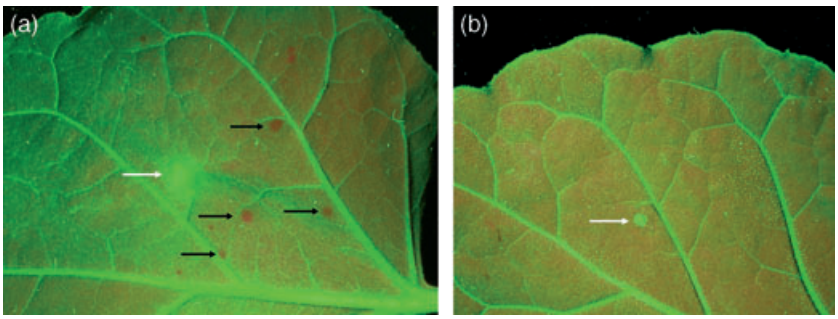

Figure 7. Induction of SSRS.
(a) Agro-infiltration of agrobacteria carrying an antisense GFP construct were able to induce SSRS 4–6 weeks post-infiltration in the infiltrated area of 16c leaves. A control agro-infiltration with a construct lacking GFP had no effect on GFP expression. White arrows mark the sites of injection, black arrows the largest silenced spots induced. Infiltration of sense GFP-transcribing constructs driven by a 35S promoter even at low concentrations did not have the same effect, although rarely a single spot did appear in the infiltrated zone (not shown).

spreads systemically would be activated. It is possible to adjust the above model to take into account not only quantitative but also qualitative issues of the silencing trigger. In that context, the amount of GFP target mRNA may also play a role. If its concentration is high, the likelihood of generation of secondary siRNA increases. That would explain why type I plants with a high steady-state concentration of GFP mRNA enter the systemic pathway after the induction of SSRS, while type II plants do not. Such a model would allow for multiple 'safety nets' in order to avoid over-reaction to relatively minor threats. In the proposed model, the key factor is the trigger molecule, which may be an aberrant RNA. It is possible that once a threshold is exceeded, an RdRP would be induced. This would generate the complementary strand of the targeted molecule creating a double-stranded RNA, and thus initiating silencing.

Experimental procedures

Plant transformations

The *pBIN* 35S-*mGFP4* construct (Haseloff *et al.*, 1997) was kindly provided by Jim Haseloff (Cambridge University, Cambridge, UK) and was used for all plant transformations. *Agrobacterium tumefaciens* strain LBA4044 was transformed with the *pBIN* 35S-*mGFP4* plasmid by tri-parental mating (Ditta *et al.*, 1980). *N. benthamiana* transformations were performed as described previously for *N. tabacum* (Kalantidis *et al.*, 2002).

All infections were done mechanically, using infectious sap. The P19 suppressor gene of cymbidium ring spot virus (Havelda *et al.*, 2003; Lakatos *et al.*, 2004) under a 35S promoter (35S P19-CymRSV in agrobacteria of the C58C1 strain) was donated by J. Burgyan (ABC, Gödöllő, Hungary). The HC-Pro TEV was donated by J.J. Lopez-Moya (CSIC, Barcelona, Spain).

Plant growth conditions

Explants and plants were grown at 25°C (day) 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} sec^{-1}. Photosynthetically active radiation (PAR) plantlets were transferred to the greenhouse at a controlled temperature of 23°C.

To test for the effect of growth conditions on spontaneous silencing induction, plants with at least six leaves were then transferred to be grown under three additional growth regimes: (i) 26°C 'day', 21°C 'night' in the growth room; (ii) 23°C for 16 h, 18°C for 8 h but continuously in darkness in the growth chamber; (iii) 28°C 'day', 22°C 'night' in the greenhouse.

Phenotypic analysis and agro-infiltration

Microscopic detection of GFP was performed using an inverted fluorescence microscope exactly as described by (Haseloff *et al.*, 1997) using an EGFP filter (Zeiss, Oberkochen, Germany) with an excitation spectrum of 450–490 nm and at an emission band pass of 515–565 nm. Confocal microscopy was conducted using a Bio-Rad Radiance 2100 system (Bio-Rad, Hercules, CA, USA). A hand-held 1000 W long-wavelength UV lamp (B1000AP; Ultraviolet Products, Upland, CA, USA) was also used for routine monitoring of transgenic shoots and plants. Silenced spots were removed from leaves by pressing a Pasteur pipette onto them, creating a circular hole with a standard diameter of approximately 1.6 mm. Agro-infiltration was performed as described previously (Schob *et al.*, 1997).

Grafting experiments

Top grafting of *N. benthamiana* plants was performed as described previously (Crete *et al.*, 2001). Scions had most of their leaves removed upon grafting and were kept covered to avoid drying for approximately one week.

Northern blot analysis

Northern analyses for both transcript and siRNA detection were performed as described previously (Kalantidis *et al.*, 2002; Papaefthimiou *et al.*, 2001).

DNA probes were labelled by random prime labelling (Invitrogen, Carlsbad CA, USA) and riboprobes were transcribed according to standard procedures (Papaefthimiou *et al.*, 2001). Hybridization to U1 RNA (a 156 nucleotide RNA of the spliceosomal snRNP complexes) using a potato U1 antisense probe was used as an internal standard to control RNA loading in short RNA Northern hybridizations.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Southern hybridization of representative type I, type II and type III lines. Lanes 1 and 5, type I lines (12.1 and 6.3 respectively), lanes 2 and 4 type II lines (5.3 and 5.1 respectively), lane 6 type III line (line 4.4) and lane 3 wt. Lane 7 size markers. Samples were digested with *Sac*I, separated on a 0.8% gel before transferring to the membrane. A DNA GFP full sequence probe was used.

Figure S2. GFP expression in different transgenic lines of *Nicotiana benthamiana*. The top shows the different GFP fluorescence in representative plants of transgenic lines of types I to III under UV light. 5.3 is a type II line carrying a large amount of SSRS spots. Plants of individual lines as indicated were analysed for levels of GFP mRNA in a Northern blot. The expression levels of GFP mRNA vary between different types of transgenic line, but also within each type (compare 12.1, 6.3 and 6.4). With the exception of type III line 4.4 which shows silencing very early, RNA was extracted from young, non-silenced and spot-less leaves. The lower panel shows the gel prior to blotting and stained with ethidium bromide to control equal loading.

Figure S3. Phenotype of representative GFP transgenic plants. Left, type I plants undergoing systemic silencing; centre, type II plant with spots but not systemic silencing and right, type III plant, fully silenced.

Figure S4. Silencing of *GFP* can be induced normally in type II plants by agroinfiltration of a construct transcribing ds *GFP* RNA. White arrow indicates agroinfiltrated leaf, black arrow initiation of silencing in newly developed leaf.

Figure S5. Assessment of the silencing transmition potential of type I and type II plants.

(a) Grafting of 16c scion on type I plant (line 12.1); spreading of silencing in the scion could be observed about 2–3 weeks later.

(b) Grafting of 16c scion on type II plant (line 5.3); no silencing of GFP expression detectable.

(c) Grafting on type II scion (line 5.3) on type I rootstock (line 12.1), spreading of silencing observed two weeks later.

Figure S6. Short range silencing spots can be also induced by overexpression of sense GFP. Agroinfiltration of bacteria carrying a sense GFP transcribing construct, in mesophyl cells of the stably expressing GFP line 16c, can sometimes induce silenced spots (yellow arrow). The characteristic red/silenced zone around the agroinfiltrated area is indicated here (white arrow) (Himber *et al.*, 2003).

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