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Arabidopsis Mutants Impaired in Cosuppression

Taline Elmayan, Sandrine Balzergue, Fabrice Béon, Violaine Bourdon, Jérémy Daubremet, Yvette Guénet, Philippe Mourrain, Jean-Christophe Palauqui, Samantha Vernhettes, Tiphaine Vialle, Katia Wostrikoff, and Hervé Vaucheret[1]

Laboratoire de Biologie Cellulaire, INRA, 78026 Versailles Cedex, France

Post-transcriptional gene silencing (cosuppression) results in the degradation of RNA after transcription. A transgenic Arabidopsis line showing post-transcriptional silencing of a 35S–*uidA* transgene and *uidA*-specific methylation was mutagenized using ethyl methanesulfonate. Six independent plants were isolated in which *uidA* mRNA accumulation and β-glucuronidase activity were increased up to 3500-fold, whereas the transcription rate of the 35S–*uidA* transgene was increased only up to threefold. These plants each carried a recessive monogenic mutation that is responsible for the release of silencing. These mutations defined two genetic loci, called *sgs1* and *sgs2* (for suppressor of gene silencing). Transgene methylation was distinctly modified in *sgs1* and *sgs2* mutants. However, methylation of centromeric repeats was not affected, indicating that *sgs* mutants differ from *ddm* (for decrease in DNA methylation) and *som* (for somniferous) mutants. Indeed, unlike *ddm* and *som* mutations, *sgs* mutations were not able to release transcriptional silencing of a 35S–*hpt* transgene. Conversely, both *sgs1* and *sgs2* mutations were able to release cosuppression of host *Nia* genes and 35S–*Nia2* transgenes. These results therefore indicate that *sgs* mutations act in *trans* to impede specifically transgene-induced post-transcriptional gene silencing.

INTRODUCTION

Plant transgenes can be silenced at either the transcriptional or post-transcriptional level (reviewed in Dougherty and Parks, 1995; Matzke and Matzke, 1995; Baulcombe, 1996; Depicker and Van Montagu, 1997; Stam et al., 1997). Transcriptional silencing involves promoter methylation and structural changes in chromatin (Ye and Signer, 1996) and thus resembles X-inactivation and parental imprinting in mammals (Barlow, 1995; Kelley and Kuroda, 1995). It also resembles position effect variegation (PEV) in Drosophila, although Drosophila lacks methylation (Dorer and Henikoff, 1994). Post-transcriptional silencing results in a strong reduction of mRNA accumulation in the cytoplasm without significant changes in the rate of transcriptional initiation in the nucleus. It can affect the expression of transgenes and homologous host genes, a phenomenon referred to as cosuppression (chalcone synthase, Van Blokland et al., 1994; glucanase, de Carvalho Niebel et al., 1995; chitinase, Kunz et al., 1996; nitrate reductase, Vaucheret et al., 1997). Post-transcriptional silencing can also affect the expression of transgenes sharing no sequence homology with host genes (neomycin phosphotransferase [*nptII*] from the bacterial transposon Tn*903* [Ingelbrecht et al., 1994], *rolB* from Agrobacterium [Dehio and Schell, 1994], and *uidA* from *Escherichia coli* [Elmayan and Vaucheret, 1996]). Cosuppression of homologous host genes and trangenes has been described mainly in plants, with the exception of one report in Neurospora (Cogoni et al., 1996) and one report in Drosophila, although in this latter case it has not been shown whether silencing occurs at the transcriptional or post-transcriptional level (Pal-Bhadra et al., 1997).

Various hypotheses have been proposed to explain the mechanism(s) of post-transcriptional silencing in plants. A biochemical switch model was proposed in which mRNA turnover is increased when a product of gene expression reaches a threshold concentration due to the use of a strong promoter (Meins, 1989; Dehio and Schell, 1994; Smith et al., 1994). However, cosuppression was also reported by using promoterless transgenes (Van Blokland et al., 1994). Therefore, an ectopic pairing model was proposed in which interactions between host genes and transgenes or between transgene copies lead to the production of aberrant RNA that activates a mechanism of sequence-specific RNA degradation (Baulcombe and English, 1996; English et al., 1996). However, post-transcriptional silencing of a transgene that does not share homology with the host genome was reported in haploid plants carrying a single copy of the transgene (Elmayan and Vaucheret, 1996), thus indicating that the presence of multiple copies is not always required. Finally, an autoregulatory degradative model was proposed

[1]To whom correspondence should be addressed. E-mail vauchere @versailles.inra.fr; fax 33-1-30-83-30-99.

in which silencing occurs by means of pairing-cleavage cycles between mRNA and shorter (aberrant) poly(A)$^-$ RNA (Metzlaff et al., 1997). Therefore, various situations might exist that lead to post-transcriptional gene silencing, including overtranscription, ectopic pairing, and/or the production of aberrant RNAs.

Infection of transgenic plants by RNA viruses that replicate in the cytoplasm can be inhibited if the virus shares homology with a transgene silenced post-transcriptionally, suggesting some relationship between gene silencing and plant defense mechanisms (Lindbo et al., 1993; Smith et al., 1994; Mueller et al., 1995; English et al., 1996; Sijen et al., 1996; Tanzer et al., 1997). Recently, a natural case of post-transcriptional silencing was reported in nontransgenic plants of kohlrabi (*Brassica oleracea gongylodes*). Plants infected by the double-stranded DNA pararetrovirus cauliflower mosaic virus (CaMV) initially developed systemic symptoms but subsequently recovered due to loss of virus. No change in transcription rate of CaMV RNAs was observed, although these RNAs failed to accumulate (Covey et al., 1997). In addition, infection of nontransgenic *Nicotiana clevelandii* plants by the single-stranded RNA tomato black ring nepovirus (strain W22) can induce a resistance mechanism that is similar to transgene-induced silencing (Ratcliff et al., 1997). Moreover, plants subsequently become insensitive to infection by a potexvirus in which W22 sequences have been cloned, whereas they are sensitive to infection by the wild-type potexvirus. This similarity between viral defense and gene silencing supports the idea that plants can combat infection by gene silencing, thus suggesting a natural role for this phenomenon. Nevertheless, the genes governing these silencing mechanisms in plants are still unknown.

Approximately 120 mutants displaying either an increase or a decrease of PEV have been characterized in Drosophila (reviewed in Karpen, 1994). Several of the corresponding genes have been cloned. Dominant mutations that suppress or enhance PEV encode either chromatin proteins or factors that directly alter chromatin structure. In plants, a few mutants have been identified, but none of the corresponding genes has been cloned. Arabidopsis mutants showing a release of transcriptional silencing have been identified recently (Mittelsten Scheid et al., 1998). These mutants define at least two genetic loci called *som* (for somniferous). *som* mutants show a decrease in the methylation of repeated sequences of the genome, as do *ddm* mutants (for decrease in DNA methylation). Indeed, *som1* is allelic to *ddm1.* Arabidopsis mutants showing an increase in the triggering of post-transcriptional silencing have also been isolated (Dehio and Schell, 1994). These mutants define two genetic loci called *egs* (for enhancer of gene silencing).

In this work, we discuss the isolation of plant mutants totally impaired in the triggering of post-transcriptional silencing. These Arabidopsis mutants define two genetic loci called *sgs* (for suppressor of gene silencing) because they have the opposite effect of *egs* mutations. The *sgs* mutants do not show a decrease in the methylation of repeated sequences of the genome and thus differ from *ddm* and *som* mutants. Conversely, they should be affected in genes similar to those affected in quelling-defective (*qde*) mutants impaired in post-transcriptional transgene-induced gene silencing that have been identified recently in Neurospora (Cogoni and Macino, 1997).

RESULTS

Identification of Transgenic Arabidopsis Lines Showing Post-Transcriptional Silencing of a 35S–*uidA* Transgene

We reported previously that the bacterial *uidA* coding sequence encoding *E. coli* β-glucuronidase (GUS) cloned between the CaMV 35S promoter and the terminator sequences of the pea ribulose bisphosphate carboxylase small subunit *rbcS-9C* gene undergoes post-transcriptional silencing in all of the transgenic tobacco lines, irrespective of transgene copy number and chromosomal position (Elmayan and Vaucheret, 1996). Eleven transgenic lines carrying a single transgene locus were analyzed. We showed that these lines belong to two groups. Two lines belong to the L group (low expressers), that is, they show a high level of transcription of the 35S–*uidA* transgene and a low level of *uidA* mRNA accumulation and GUS activity in both homozygous and hemizygous plants. Nine lines belong to the H^c group (high conditional expressers), that is, they show a low level of *uidA* mRNA accumulation and GUS activity when the plants are homozygous for the transgene and a high level of *uidA* mRNA accumulation and GUS activity when they are hemizygous. No lines accumulate a high level of *uidA* mRNA and GUS activity whenever the plants are homozygous or hemizygous for the transgene, thus indicating that this construct is highly prone to silencing. In both the L and H^c groups, silencing was mitotically heritable but meiotically reversible: it occurred in each generation during the development of the plants, and resetting of transgene expression occurred at meiosis.

The same transgene linked to an *nptII* selectable marker was introduced into Arabidopsis ecotype Columbia (Col-0). Ten transgenic lines carrying the transgene inserted at a single locus were selected, and homozygous descendants were identified. Among these 10 homozygous lines, four showed at each generation a high level of GUS activity in young seedlings (between 2000 and 6000 nmol of 4-methylumbelliferone [MU] per min per μg of protein) followed by a strong and rapid decrease of GUS activity during development, reaching a very low level (<1 nmol of MU per min per μg of protein in line L1) in adult leaves (Figure 1). Silencing occurred during early development in both homozygous and hemizygous plants (data not shown), thus indicating that these four plants belong to the L group. Molecular analysis of these four L lines revealed that they all carry multiple copies of the T-DNA (data not shown), which is consistent with

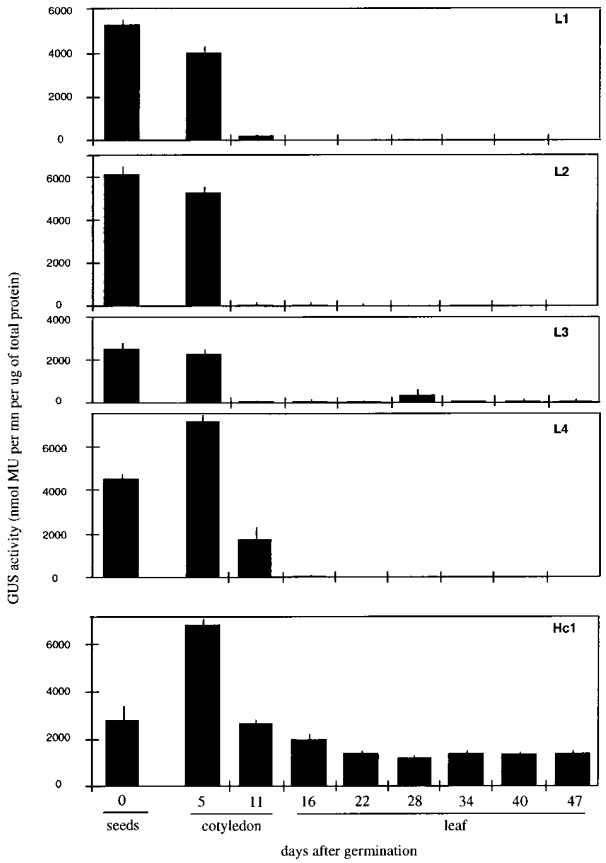

Figure 1. Evolution of GUS Activity in Young Seedlings and Mature Plants.

Proteins were extracted from seeds (day 0), cotyledons (days 5 and 11), or leaves (days 16, 22, 28, 34, 40, and 47) of four homozygous L lines (low expressers; L1, L2, L3, and L4) and one homozygous H^c line (high conditional expresser; H^c1) grown in the greenhouse. GUS activity (in nanomoles of MU per minute [mn] per microgram of total protein) was measured by fluorometric analysis using 0.5 μg of total protein. Bars indicate standard errors of the mean.

the observation that tobacco L lines also carry multiple copies of the T-DNA (Elmayan and Vaucheret, 1996).

The six other lines belong to the H^c group, that is, they showed silencing in homozygous descendants but not in hemizygous descendants (data not shown). However, they behaved differently than did the tobacco H^c lines. For instance, a bulk analysis of homozygous plants derived from line H^c1 revealed GUS activity of ~1000 nmol of MU per min per μg of protein after 1 month of growth (Figure 1). However, a plant-by-plant analysis revealed that not all of the plants expressed the transgene at a high level. Indeed, 15% of the plants showed a low level of expression (<1 nmol of MU per min per μg of protein) at the adult stage. When seeds were harvested from plants with either high or low levels of expression and sown for analysis in the next generation, the same frequency of ~85% high- and 15% low-expressing plants was observed, indicating that silencing affects only 15% of the homozygous individuals in each generation. Such transformant-specific frequencies have already been observed in different cases of cosuppression (chitinase, Kunz et al., 1996; nitrate reductase, Vaucheret et al., 1997).

Expression of the 35S–*uidA* transgene was monitored by RNA gel blot and run-on analysis. These experiments were conducted with adult leaves of plants derived by selfing from a homozygous descendant of the H^c1 line showing high GUS activity and with adult leaves of plants derived by selfing from a homozygous descendant of the L1 line. This line was chosen from among the four L lines for two reasons. (1) It exhibits a simple molecular pattern of a direct repeat of two T-DNAs, whereas the other L lines exhibit more complex patterns (data not shown). (2) It has the lowest level of GUS activity (<1 nmol of MU per min per μg of protein). Figures 2A to 2D show that *uidA* mRNAs accumulated at a high level in the H^c1 line, whereas accumulation was below the level of detection in the L1 line, thus correlating with the observed GUS activity in these plants. However, the 35S–*uidA* transgene was transcribed in the nucleus at a high level in both the H^c1 and L1 lines, thus indicating that silencing in line L1 is post-transcriptional. No antisense *uidA* RNAs could be detected, suggesting that silencing does not result from an antisense effect, which is consistent with the observation made in post-transcriptionally silenced tobacco lines carrying the same transgene (Elmayan and Vaucheret, 1996). The transcription rate was slightly higher in L1 compared with H^c1, suggesting that H^c1 is just below the threshold level of transcription required to trigger silencing, whereas L1 is above this level.

Identification of Arabidopsis Mutants Impaired in 35S–*uidA* Silencing

Five hundred seeds of the homozygous L1 line were mutagenized with ethyl methanesulfonate (EMS). Plants were allowed to self-fertilize, and seeds were harvested in bulks from five mutagenized plants. Fifty seeds from each of the 100 bulks were sown in the greenhouse. Because GUS activity in line L1 is <1 nmol of MU per min per μg of protein after 1 month of growth, plants expressing high levels of GUS activity were screened at this stage of development. Seven plants with GUS activity between 1000 and 4000 nmol of MU per min per μg of protein were identified in seven independent bulks. These plants were allowed to self-fertilize. For each of them, 50 seeds were sown in the greenhouse, and GUS activity was monitored throughout each plant's life. Six plants showed high expression of GUS activity in all of their progeny throughout development (M1, 1600 ± 200; M2, 3500 ± 250; M3, 2900 ± 300; M4, 2750 ± 200;

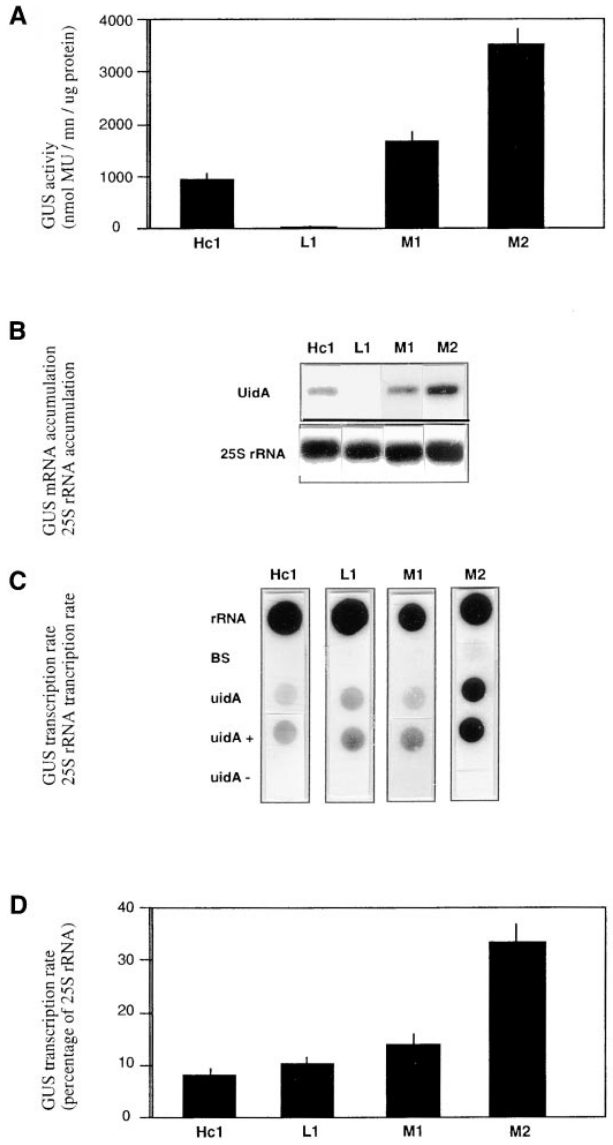

Figure 2. Expression Analysis of the 35S–*uidA* Transgene in Nonsilenced Plants Derived from the H^c1 Line, in Silenced Plants Derived from Line L1, and in the Mutants M1 (Allele *sgs1-1*) and M2 (Allele *sgs2-1*) Obtained by EMS Mutagenesis of the L1 Line.

(A) GUS activity (in nanomoles of MU per minute [mn] per microgram of total protein) was measured by fluorometric analysis using 0.5 μg of protein extracted from leaves of adult plants grown in the greenhouse. Four experiments were performed using independent plants. The average GUS activity is shown. Error bars correspond to standard deviation.

(B) *uidA* mRNA steady state level was determined by RNA gel blot analysis using 4 μg of total RNA extracted from leaves of adult plants and hybridized with the *uidA* coding sequence and with the 25S rRNA as the control.

(C) The *uidA* nascent transcript level was determined by run-on experiments using labeled RNA extracted from leaves of adult plants that were hybridized with slot blots containing 2 μg each of the 25S rRNA–containing plasmid (rRNA, double stranded), empty pBluescript KS+ plasmid (BS, single stranded; Stratagene), and *uidA*-containing plasmid (uidA, double stranded; uidA+, antisense single stranded; and uidA−, sense single stranded).

(D) The average GUS transcription rates (percentage of 25S rRNA) were determined from four experiments using independently isolated nuclei. Error bars correspond to standard deviation.

M5, 3200 ± 400; M6, 3100 ± 350 nmol of MU per min per μg of protein), indicating that the impairment of silencing in these plants is heritable. The remaining plant (M7) showed a delay in the triggering of silencing compared with the L1 line, but all of its progeny became silenced after 6 weeks of growth.

Mutations Are Recessive and Monogenic and Define Two Genetic Loci

Table 1 summarizes the genetic analysis of the six mutants stably expressing a high level of GUS activity. Hybrids obtained by crossing the mutants with a wild-type plant or with the L1 line were silenced for GUS activity, indicating that the six mutants each carry a recessive mutation. Hybrids between the mutants and the L1 line were allowed to self-fertilize. For each mutant, 100 seeds were sown in the greenhouse, and GUS activity was monitored after 1 month of growth. A 3:1 ratio of silenced-to-nonsilenced plants was observed in each case (M1, 74:26; M2, 72:28; M3, 75:25; M4, 72:28; M5, 73:27; M6, 77:23), suggesting that the six mutants each carry a single recessive nuclear mutation. Complementation tests were performed by reciprocally crossing the six mutants. These tests allowed us to define two genetic loci called *sgs.* One mutant (M1) defined the locus *sgs1* (allele *sgs1-1*). The remaining five mutants (M2, M3, M4, M5, and M6) defined the locus *sgs2* (alleles *sgs2-1*, *sgs2-2*, *sgs2-3*, *sgs2-4*, and *sgs2-5*).

Mutations Do Not Affect the T-DNA

To determine whether the mutations affect the T-DNA or unlinked loci acting in *trans*, we performed both molecular and genetic analyses. DNA gel blot analysis revealed no significant modifications of the structure of the transgene locus in the six mutants (data not shown). Figure 3 summarizes how the T-DNA was genetically segregated away from the *sgs* mutations in M1 (allele *sgs1-1*) and M2 (allele *sgs2-1*). Both mutants expressing GUS at a high level (GUS+) were crossed with wild-type plants. Silenced (GUS−) F_1 hybrids were allowed to self-fertilize. F_2 seeds were sown on kanamycin. Among the population of F_2 kanamycin-resistant (Km^r) plants, those that were homozygous for the mutation and hemizygous for the T-DNA were identified as having a 3:1 ratio of Km^r GUS+ to kanamycin-sensitive (Km^s) GUS−

Table 1. Crosses and Complementation Analysis of EMS Mutants Impaired in Silencing

	Male Parent[a]							
Female Parent	WT[b]	L1[c]	M1[d]	M2[d]	M3[d]	M4[d]	M5[d]	M6[d]
L1	0/40	0/100	ND[e]	ND	ND	ND	ND	ND
M1	0/5	0/13	50/50	0/20	0/20	ND	0/20	0/20
M2	0/5	0/13	ND	50/50	20/20	ND	ND	ND
M3	0/5	0/20	ND	ND	50/50	ND	ND	ND
M4	0/5	0/4	0/20	20/20	20/20	50/50	20/20	20/20
M5	0/5	0/20	ND	20/20	20/20	ND	50/50	ND
M6	0/5	0/13	ND	20/20	8/8	ND	20/20	50/50

[a] The values given are the number of GUS-positive seedlings after 1 month of growth per total number of seedlings.
[b] WT, wild type.
[c] L1, silenced transgenic line used for mutagenesis.
[d] M1 (*sgs1*), M2, M3, M4, M5, and M6 (*sgs2*) mutants.
[e] ND, not determined.

plants. We confirmed that these GUS− F_3 descendants were homozygous for the mutation and lacked the T-DNA by selfing and by crossing with the mutant from which they were derived. Plants derived from self-fertilization (F_4) were all Km^s GUS−, whereas plants derived from a cross with the mutant were all Km^r GUS+, thus confirming that these GUS− F_3 plants lacking the T-DNA were homozygous for the mutation. The identification of such plants indicates, therefore, that *sgs* mutations do not affect the T-DNA and that they act in *trans.*

Expression of the 35S–*uidA* Transgene in *sgs1* and *sgs2* Mutants

Expression of the 35S–*uidA* transgene was analyzed in *sgs1* and *sgs2* mutants and was compared with line L1. GUS activity was monitored by fluorometric measurements, RNA accumulation by RNA gel blot analysis, and transcription rate by run-on experiments (Figure 2). GUS activity was ~1 nmol of MU per min per μg of protein in line L1, whereas it was 1600 ± 200 nmol of MU per min per μg of protein in M1 (allele *sgs1-1*) and 3500 ± 250 nmol of MU per min per μg of protein in M2 (allele *sgs 2-1*). *uidA* mRNA was undetectable in line L1, whereas it accumulated to a high level in *sgs* mutants, approximately twofold more in M2, M3, M4, M5, and M6 when compared with M1 (Figure 2 for M1 and M2; data not shown for the other *sgs2* alleles). The transcription rate was slightly higher in *sgs* mutants when compared with L1. A 1.3-fold increase was observed in M1 compared with L1, whereas a 3.4-fold increase was observed in M2 compared with L1. This result indicates that the release of post-transcriptional silencing does not result from a small reduction of transcription below the level required to trigger silencing. Therefore, the high increase in both *uidA* mRNA accumulation and GUS activity indicates that the release of silencing in *sgs* mutants results from a change at the post-transcriptional level.

sgs1 and *sgs2* Mutations Act in *Trans* to Impede Cosuppression of *Nia* Host Genes and 35S–*Nia2* Transgenes

We investigated whether *sgs1* and *sgs2* mutations specifically impede *uidA* silencing or whether they can impede other post-transcriptional silencing phenomena, including the so-called cosuppression of transgenes and homologous host genes. For this purpose, a 35S–*Nia2* transgene consisting of the nitrate reductase *Nia2* gene cloned downstream of the 35S promoter (Wilkinson and Crawford, 1991) was linked to a hygromycin phosphotransferase (*hpt*) selectable marker and introduced via Agrobacterium into wild-type plants (Col-0), the L1 line, and the mutants M1 (allele *sgs1-1*) and M2 (allele *sgs2-1*). Cosuppression of *Nia* host genes and transgenes was observed in 19 of 20 primary transformants obtained with Col-0 plants (Table 2). Because cosuppression affected all of the homozygous descendants of the unique nonsilenced transformant (Col-2a3), cosuppression of nitrate reductase appears to be 100% efficient in Arabidopsis. Cosuppression was observed in all 18 transformants obtained with line L1 (Table 2), indicating that cosuppression is equally efficient in wild-type plants and in transgenic plants that are already silenced for a 35S–*uidA* transgene. Conversely, cosuppression was not observed among the eight M1 and 18 M2 primary transformants or in their progeny (Table 2). To test whether *sgs1* and *sgs2* mutations can also release a cosuppressed state, crosses were performed between a silenced homozygous plant derived from transformant Col-2a3 and the *sgs1* and *sgs2* mutants. If the 35S–*Nia2* transgene and the *sgs* mutations were to segregate independently, 6.25% of the F_2 descendants should be homozygous for the 35S–*Nia2* transgene and nonsilenced (i.e., homozygous for the *sgs* mutation). Seven of 125 (5.6%) F_2 descendants from the cross Col-2a3 × *sgs1* and six of 83 (7.2%) F_2 descendants from the cross Col-2a3 × *sgs2* were homozygous for the 35S–*Nia2* transgene and nonsilenced,

indicating that *sgs1* and *sgs2* mutations act in *trans* to release cosuppression of *Nia* genes.

sgs1 and *sgs2* Mutations Do Not Impede Transcriptional Silencing of a 35S–*hpt* Transgene

To test whether *sgs* mutants were affected specifically in post-transcriptional silencing, we crossed both M1 and M2 mutants with the homozygous line A carrying a transcriptionally silenced 35S–*hpt* transgene (Mittelsten Scheid et al., 1998). F_1 hybrids were allowed to self-fertilize, and F_2 seeds were sown on medium supplemented with hygromycin. None of 500 seedlings was able to grow on this medium, indicating that neither *sgs1* or *sgs2* mutations are able to release transcriptional silencing, as opposed to *som* mutations, which are able to release silencing in line A (Mittelsten Scheid et al., 1998). Therefore, *SGS* genes seem to be involved specifically in post-transcriptional silencing.

sgs1 and *sgs2* Mutations Distinctly Affect the Methylation State of the 35S–*uidA* Transgene but Not That of Repetitive Host Sequences

The methylation state of the 35S–*uidA* transgene was monitored by using the methylation-sensitive enzymes MspI and HpaII (Figures 4A to 4D). No methylation was observed in the *uidA* coding sequence of line H^c1. Methylation of HpaII-MspI sites was found in the central part and 3′ end but not in the 5′ end of the *uidA* coding sequence of lines L1, L2, L3, and L4 (Figure 4 and data not shown). Similar results were reported previously in tobacco (English et al., 1996). The methylation pattern of line L1 did not vary from one plant to

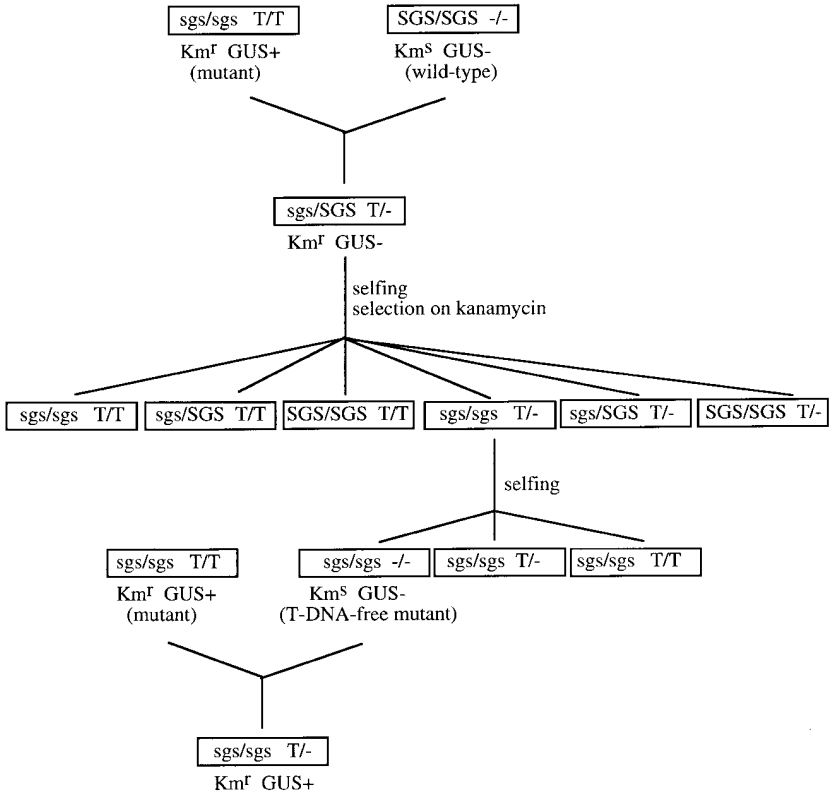

Figure 3. Scheme for Segregation of the T-DNA from the *sgs* Mutations.

We designated *sgs* as the mutant allele and *SGS* as the wild-type allele, T as the T-DNA, and (−) as the T-DNA–free locus. *sgs* mutants (*sgs*/*sgs* T/T) expressing GUS (GUS+) were crossed with a wild-type plant (*SGS*/*SGS* −/−), resulting in F_1 hybrids (*sgs*/*SGS* T/−), which are silenced (GUS−) because *sgs* mutations are recessive and because silencing affects the 35S–*uidA* transgene in both homozygous and hemizygous plants. F_1 hybrids were allowed to self-fertilize, and F_2 seeds were sown in vitro on a medium supplemented with kanamycin. Km^r plants were transferred to the greenhouse and allowed to self-fertilize. F_2 plants that are homozygous for the mutation and hemizygous for the T-DNA (*sgs*/*sgs* T/−) were identified as having a 3:1 ratio of Km^r GUS+ to Km^s GUS− plants. GUS− F_3 descendants were crossed with the mutant from which they originally derived (*sgs*/*sgs* T/T). Hybrids were all Km^r GUS+, thus confirming that these F_3 GUS− plants are homozygous for the mutation and lack the T-DNA (*sgs*/*sgs* −/−).

another, indicating that methylation in the center and 3′ end of the *uidA* sequence correlates with post-transcriptional silencing. No methylation was found in the 35S promoter of line H^c1 and L1 (Figure 4A). Conversely, lines L2, L3, and L4 showed partial methylation in the 35S promoter (data not shown). The presence of multiple copies of the T-DNA integrated in a complex pattern in lines L2, L3, and L4 could explain the partial methylation of the 35S promoter. Conversely, line L1 carries only a direct tandem of two T-DNA copies, whereas line H^c1 carries one complete T-DNA copy and one truncated copy (data not shown). Indeed, the absence of methylation in the 35S promoter was an additional reason for the choice of line L1 for the EMS mutagenesis.

Mutant M2 (allele *sgs2-1*) showed complete demethylation of CNG sites (analysis of MspI digests) and partial demethylation of CG sites (analysis of HpaII digests) within the *uidA* sequences (Figures 4B to 4D)—a result also found with the other *sgs2* mutants (data not shown). No difference in methylation was found between *sgs2* mutants and line L1 in the 35S sequences. Conversely, partial methylation was found in the 35S promoter of the mutant M1 (allele *sgs1-1*), leading to a pattern resembling those of lines L2, L3, and L4. Methylation in mutant M1 was unchanged at the 5′ end of *uidA* and partially decreased at CNG sites in the center and 3′ end of *uidA*, as compared with the L1 line. No significant differences were observed between M1 and L1 at CG sites. Similar results were obtained using DNA extracted from either floral buds or leaves and using DNA extracted from selfed mutants or backcrossed descendants. Therefore, these results indicate that the state of methylation of the 35S–*uidA* transgene is distinctly affected in these two *sgs* genetic backgrounds.

To test whether *sgs1* and *sgs2* mutations affect the overall level of methylation of the genome or whether the modification of transgene methylation is an indirect consequence of the release of silencing, we analyzed the methylation pattern of two repetitive methylated sequences of the genome. Methylation was monitored by digestion of the DNA of Col-0, L1, M1, and M2 lines and of the *ddm1* mutant, showing a decrease in DNA methylation (Vongs et al., 1993) with the methylation-sensitive enzyme HpaII and hybridization with a 180-bp centromere repeat (Figure 5) or a 5S rDNA probe (data not shown). Results indicate that the level of methylation of repetitive sequences of the genome is unaffected in both *sgs1* and *sgs2* mutants, thus suggesting that *sgs1* and *sgs2* mutants are not affected in the general control of methylation.

Table 2. Efficiency of Nitrate Reductase Silencing by Cosuppression

Transformed Line[a]	Number of Transformants	Number of Silenced Transformants	Cosuppression Frequency (%)
WT	20	19 + 1[b]	100
L1	18	18	100
M1	8	0	0
M2	18	0	0

[a] Plants that show cosuppression of host *Nia* genes after introduction of the 35S–*Nia2* transgene become chlorotic on medium containing nitrate as the sole source of nitrogen but can grow on medium supplemented with 10 mM glutamine. WT, wild type; L1, silenced transgenic line used for mutagenesis; M1 (allele *sgs1-1*) and M2 (allele *sgs2-1*), mutants.

[b] One transformant showed cosuppression only in homozygous descendants.

DISCUSSION

Initially, two hypotheses were proposed to explain transgene-induced post-transcriptional gene silencing in plants. Silencing may result from particular DNA–DNA interactions between homologous gene copies or from the overproduction of RNAs above a threshold level due to the use of strong promoters (reviewed in Dougherty and Parks, 1995; Matzke and Matzke, 1995; Baulcombe, 1996; Depicker and Van Montagu, 1997; Stam et al., 1997). These two hypotheses may not be exclusive if we consider that in both situations, a signal that triggers specific RNA degradation is produced. An RNA-mediated RNA degradation pathway has been proposed (Metzlaff et al., 1997) in which aberrant RNAs are involved in cycles of aberrant RNA–mRNA pairing between complementary sequences followed by endonucleolytic cleavages. Therefore, aberrant RNA may participate in various silencing events involving different transgene constructs. Indeed, aberrant RNA could be produced by gene repeats when they are involved in particular DNA–DNA interactions, thus explaining the dependence on transgene repeats to trigger cosuppression of host genes by promoterless homologous transgenes (Van Blockland et al., 1994; Stam et al., 1997). Alternatively, the use of strong promoters may increase the amount of aberrant RNA spontaneously produced by the transgene because of transcriptional stops or errors, thus explaining the dependence of cosuppression on transgene promoter strength (Que et al., 1997) and on transgene transcription (Vaucheret et al., 1997).

We reported previously that a strongly expressed 35S–*uidA* transgene can trigger post-transcriptional silencing very efficiently when introduced into tobacco, irrespective of the transgene copy number (Elmayan and Vaucheret, 1996). All 11 transgenic lines that we analyzed showed silencing. L lines showed silencing irrespective of the allelic state of the transgene, whereas H^c lines showed silencing only when the transgene was in a homozygous state. Introduction of the same transgene into Arabidopsis led to post-transcriptional silencing as efficiently as it did in tobacco. Indeed, in our analysis, all 10 lines showed silencing and belong either to the L or the H^c group, thus indicating that this particular construct is highly prone to silencing for an unknown reason.

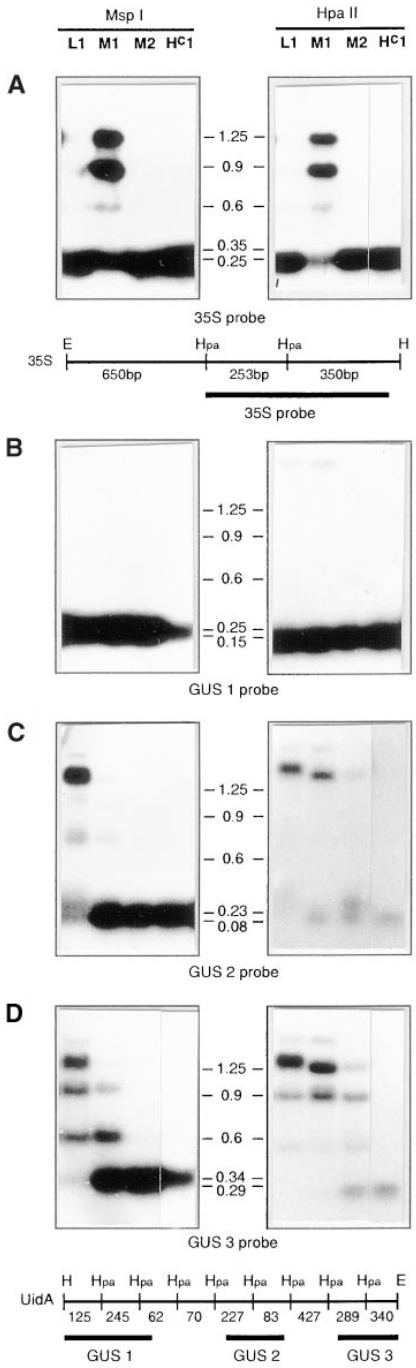

Figure 4. Methylation Analysis of the 35S–*uidA* Transgene in Silenced Plants Derived from Line L1 and the Mutants M1 (Allele *sgs1-1*) and M2 (Allele *sgs2-1*) Obtained by EMS Mutagenesis of the L1 Line and Nonsilenced Plants Derived from Line H^c1.

DNA gel blot analysis was performed using 0.5 μg of genomic DNA extracted from floral buds or leaves of adult plants digested with EcoRI, HindIII, and either MspI or its isoschizomer HpaII.
(A) Blots were probed with the 35S sequence (35S).
(B) Blots were probed with the 5′ end of the *uidA* sequence (GUS 1).
(C) Blots were probed with the central part of the *uidA* sequence (GUS 2).
(D) Blots were probed with the 3′ end of the *uidA* sequence (GUS 3).
The lengths of the expected restriction fragments are indicated on the 35S and *uidA* maps in base pairs. E, EcoRI; H, HindIII; Hpa, HpaII. Numbers in the center indicate molecular size markers in kilobases.

After mutagenesis of 500 seeds of a silenced line (line L1) with EMS, seven mutants impaired in silencing were isolated. Six of them, showing total release of silencing, carry monogenic recessive nuclear mutations that define two genetic loci called *sgs1* (one allele; M1) and *sgs2* (five alleles; M2, M3, M4, M5, and M6). The subsequent screening of 2000 mutagenized seeds confirmed that *sgs2* mutants can be isolated at a high frequency (H. Vaucheret, unpublished results), indicating that *SGS2* is a highly mutable gene. No difference in the structure of the T-DNA was observed between line L1 and these mutants. In addition, *sgs1* and *sgs2* mutations were genetically segregated from the T-DNA. Therefore, the release of silencing does not result from rearrangements and/or mutations within the T-DNA. We tested whether *sgs* mutants were specifically affected in the silencing of a 35S–*uidA* transgene or whether they were also able to resist cosuppression of a host gene. When a 35S–*Nia2* transgene was introduced into *sgs1* and *sgs2* mutants, cosuppression of *Nia* host genes and transgenes was not observed, whereas cosuppression occurred with 100% efficiency after the introduction of the same transgene into wild-type plants or into the silenced L1 plants.

These results indicate that *sgs1* and *sgs2* mutations act in *trans.* They also indicate that *sgs* mutants are impeded in cosuppression of homologous host genes and transgenes as well as post-transcriptional silencing of exogenous transgenes. Conversely, *sgs1* and *sgs2* mutations did not release transcriptional silencing of a target 35S–*hpt* transgene, unlike *som* and *ddm* mutants (Mittelsten Scheid et al., 1998). Therefore, *SGS* genes must govern specific features of transgene-induced post-transcriptional gene silencing.

Methylation of the *uidA* coding sequence was found associated with post-transcriptional silencing in both tobacco (English et al., 1996) and Arabidopsis (this study). Indeed, a strong methylation was found at CG and CNG sites of the central part and 3′ end of the *uidA* coding sequence in the silenced tobacco lines and in the Arabidopsis L1, L2, L3, and L4 lines. Conversely, no methylation was detected in nonsilenced tobacco lines and in the Arabidopsis line H^c1. However, methylation at these loci could not be strictly compared because they differ in T-DNA copy number and structural arrangement. Precise methylation analysis could be done by analyzing the same locus in wild-type, *sgs1*, and *sgs2* backgrounds. We observed that the methylation state of the 35S–*uidA* transgene was distinctly modified in *sgs1* and

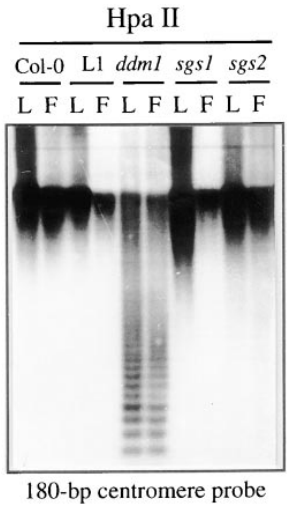

Figure 5. Methylation Analysis of Repetitive Sequences in Wild-Type Col-0, the Silenced Transgenic Line L1, the *ddm1* Mutant, and the Silencing *sgs1* (M1) and *sgs2* (M2) Mutants Obtained by EMS Mutagenesis of the L1 Line.

DNA gel blot analysis was performed using 0.5 μg of genomic DNA extracted from leaves of 25-day-old plants (L) or from floral buds of 47-day-old plants (F), digested with HpaII, and probed with the 180-bp centromere repeat.

sgs2 mutants. Mutants M2, M3, M4, M5, and M6 (*sgs2* alleles) showed no *uidA* methylation at CNG sites and a reduction of methylation at CG sites. The mutant M1 (*sgs1-1* allele) showed a reduction of *uidA* methylation at CNG sites and no reduction at CG sites. In addition, it showed partial methylation in the 35S promoter, whereas lines H^c1 and L1 or *sgs2* mutants did not show such methylation in the 35S promoter.

The subsequent screening of 2000 mutagenized seeds allowed us to isolate a second *sgs1* mutant (allele *sgs1-2*) that showed the same methylation pattern in the 35S promoter (H. Vaucheret, unpublished results), indicating that it is a reproducible feature of *sgs1* mutants. The fact that *uidA* methylation was still found in *sgs* mutants although they expressed GUS activity at a higher level than did nonsilenced nonmethylated H^c1 plants suggests that not all of the *uidA* methylation observed in the silenced L1 line can be correlated with silencing. The level of *uidA* methylation observed in the five different *sgs2* mutants might reflect the basal level of methylation of the 35S–*uidA* transgene at this locus due to the presence of a direct repeat of two T-DNAs (data not shown). An additional level of methylation might be correlated with post-transcriptional silencing, thus explaining the level of *uidA* methylation observed in the silenced L1 line.

We observed that transcription in the mutant M2 (allele *sgs2-1*) was 3.4-fold higher than in the silenced L1 line. This result suggests that transcription was slightly reduced in L1 due to silencing-related methylation in the *uidA* coding sequence. Indeed, it has been reported previously that transcription is reduced partially in *Ascobolus* as a consequence of silencing-induced methylation of the coding sequence (Barry et al., 1993). The impairment of post-transcriptional silencing and cosuppression in *sgs2* mutants may therefore impede the appearance of this silencing-related methylation, thus allowing the maximum rate of transcription of 35S-driven transgenes to be reached. This could explain why mRNAs accumulated to very high levels in an *sgs2* background compared with wild-type or *sgs1* backgrounds. Indeed, transcription in M1 was between that of the silenced L1 line (wild-type background) and of M2. The M1 mutant showed only a decrease in *uidA* methylation at CNG sites but not at CG sites. In addition, it showed a partial hypermethylation in the 35S promoter. This partial methylation in the 35S promoter might contribute to reduced transcription initiation, thus explaining why initiation was slightly lower in this background compared with an *sgs2* background.

Methylation of the 35S–*uidA* transgene was distinctly modified in *sgs1* and *sgs2* mutants. However, methylation was not modified in repetitive sequences of the genome. Therefore, *sgs* mutants differed clearly from *ddm* mutants, which showed decreased methylation in their repetitive sequences (Vongs et al., 1993; Kakutani et al., 1995), and from *som* mutants, which also showed a decreased methylation in their repetitive sequences (Mittelsten Scheid et al., 1998). In addition, *sgs* mutants did not release transcriptional silencing of the target 35S–*hpt* transgene (this study), unlike *som* mutants (Mittelsten Scheid et al., 1998).

Taken together, these results suggest that *sgs* mutants are specifically affected in post-transcriptional silencing and subsequently in silencing-related transgene methylation rather than in the general control of genome methylation and subsequently in post-transcriptional transgene silencing. If the *sgs* mutants are not directly impaired in DNA methylation (although it is not proven), they should be impaired at some other steps invoked to explain post-transcriptional gene silencing. Among these different but not exclusive steps, we can cite (1) sensing (foreign) transgene DNA in the genome, chemical modification of transgene DNA, and/or structural modification of chromatin at the transgene locus; (2) production of aberrant RNA by the transgene, sensing aberrant RNA, and degradation of aberrant RNA, mRNA, and/or mRNA–aberrant RNA duplexes; and (3) sensing and degradation of mRNA accumulated above a threshold level.

The identity and the function of plant genes involved in the control of post-transcriptional silencing are not known. In addition, the natural role they play also remains unclear. The similarity between viral defense and post-transcriptional gene silencing supports the idea that plants use the RNA degradation pathway of post-transcriptional silencing to combat virus infection (Covey et al., 1997; Ratcliff et al., 1997). The characterization of mutants impaired in post-transcriptional gene silencing will probably help to define which and how many components (if any) are common to

these two mechanisms. Both Arabidopsis *egs* and *sgs* mutants have been isolated thus far and are viable and (apparently) normal (Dehio and Schell, 1994; this study). The analysis of the response of these mutants to pathogen attacks and the cloning of the wild-type alleles corresponding to the mutated genes will provide further insights in our understanding of these fascinating processes.

METHODS

Plant Transformation, Mutagenesis, Mutant Detection, and Genetic Analysis

Transformation of *Arabidopsis thaliana* ecotype Columbia (Col-0) with *Agrobacterium tumefaciens* C58C1 (pMP90) carrying 35S–*uidA* (Elmayan and Vaucheret, 1996) or 35S–*Nia2* constructs (Wilkinson and Crawford, 1991) was performed as described by Bechtold et al. (1993). The selection of transformants was conducted in vitro on medium supplemented with 50 mg/L kanamycin or 30 mg/L hygromycin, respectively. Five hundred seeds of the homozygous transgenic L1 line were incubated for 16 hr at room temperature in 10 mL of water containing 0.4% ethyl methanesulfonate (EMS) (Sigma), washed several times with water, and planted in soil. Plants were allowed to self-fertilize, and seeds were harvested in bulks of five mutagenized plants. Seeds were sown in the greenhouse, and plants were screened for high levels of β-glucuronidase (GUS) activity after 1 month of growth. Progeny were grown in growth chambers with controlled conditions (23°C with a 16-hr-light and 8-hr-dark photoperiod at 70% humidity and with 120 $\mu E\ m^{-2}\ sec^{-1}$ lighting). The complementation analysis of mutants was done by reciprocally crossing each pair of mutants, and GUS+ seedlings were scored among the F_1 progeny after 1 month of growth in vitro.

Expression and Methylation Analysis

GUS activity (in nanomoles of 4-methylumbelliferone [MU] per minute per microgram of total protein) was measured by fluorometric analysis using 0.5 μg of protein, as described previously (Elmayan and Vaucheret, 1996).

RNA and DNA extraction and RNA and DNA gel blot analyses were performed as described previously (Elmayan and Vaucheret, 1996). Methylation analysis of the genome was performed using either a 180-bp centromere repeat or a 5S rDNA (Vongs et al., 1993; Kakutani et al., 1995) as a probe. The *ddm1* (decrease in DNA methylation) mutant was used as a control for methylation analysis (Vongs et al., 1993; Kakutani et al., 1995).

Isolation of nuclei was adapted from Dehio and Schell (1994) and Elmayan and Vaucheret (1996). Arabidopsis leaves (5 to 10 g) were ground to a fine powder in liquid nitrogen, suspended in 40 mL of buffer A (0.25 mM sucrose, 10 mM NaCl, 2% dextran T40, 10 mM Mes, pH 6.6, 5 mM EDTA, and 0.6% Triton X-100). Filtration and centrifugation were done as described previously (Dehio and Schell, 1994). The pellet was resuspended in 2 mL of the same buffer and was loaded on a step gradient of Percoll/sucrose. After centrifugation, nuclei were found in the pellet and in the interface between the sucrose layer and 80% Percoll. Nuclei were washed and resuspended in 0.2 mL of 20 mM Hepes, pH 7.2, 5 mM $MgCl_2$, 2 mM DTT, and 30% glycerol and stored at −80°C. Nuclear run-on transcription assays were performed as described previously (Elmayan and Vaucheret, 1996) with 0.2 mL of nuclei suspension.

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