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Genetics

Identification of plant genetic loci involved in a posttranscriptional mechanism for meiotically reversible transgene silencing

(gene expression regulation/modifier locus/*Arabidopsis thaliana*)

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ABSTRACT **Numerous reports describe phenomena of transgene silencing in plants, yet the underlying genetic and molecular mechanisms are poorly understood. We observed that regeneration of *Arabidopsis thaliana* plants transgenic for the *rolB* gene of *Agrobacterium rhizogenes* results in a selection for transgene silencing. Transgene silencing could be monitored in this system by reversion of the visible RolB phenotype. We report a phenotypic, molecular, and genetic characterization of a meiotically reversible transgene silencing phenomenon observed in a *rolB* transgenic line. In this line, the *rolB* gene is expressed strongly and uniformly in seedlings, but in the course of further development, the *rolB* gene is silenced erratically at a frequency that depends on the dosage of *rolB*. The silenced state is mitotically stable, while complete resetting of *rolB* gene expression occurs in seedlings of the following generation. The silencing of *rolB* correlates with a dramatic reduction of steady-state *rolB* transcripts, while *rolB* nuclear run-off transcripts are only moderately reduced. Therefore, *rolB* gene silencing seems to act predominantly at the posttranscriptional level. The process of *rolB* gene silencing was found to be affected by two extragenic modifier loci that influence both the frequency and the timing of *rolB* gene silencing during plant development. These genetic data demonstrate a direct involvement of defined plant genes in this form of gene silencing.**

The development of techniques to introduce foreign DNA into the plant genome has hastened the use of reverse genetics to study basic aspects of plant biochemistry, physiology, and development (for review, see ref. 1). Although the introduced DNA is physically transmitted as a dominant Mendelian trait (2), the expression of transgene-encoded phenotypes was found to be unstable in many cases. Several phenomena of transgene silencing have been reported (for reviews, see refs. 3–5), which correlated with transgene copy number (6), state of methylation (6, 7), homology of the transgene to endogenous genes (8, 9), and the homozygous state of the transgene (10, 11). However, our understanding of the basic mechanisms governing transgene silencing is still very limited. This is partly due to the lack of an appropriate system allowing a combined genetic and molecular study of transgene silencing. Indeed, transgene silencing was often found accidentally rather than being the primary focus of the experimental design. We introduced a suitable tester gene into *Arabidopsis* to study transgene silencing. The advantages of *Arabidopsis* as a model system for plant genetics and molecular biology are well documented (for review, see ref. 12). The *rolB* gene from the Ri-T_L DNA of *Agrobacterium rhizogenes*, when expressed by a constitutive promoter in transgenic tobacco callus, was reported to inhibit the regeneration of transgenic plants (13). In *Arabidopsis*, we took advantage of this property of the *rolB* gene to select for transgene silencing during the regeneration of transgenic plants. Furthermore, because the RolB phenotype is cell autonomous (13), it was possible to visually monitor *rolB* gene silencing in somatic tissues. We describe here a detailed phenotypic, molecular, and genetic characterization of a meiotically reversible transgene-silencing phenomenon acting predominantly at the posttranscriptional level. Mutable plant loci are shown to control this phenomenon.

MATERIALS AND METHODS

Plant Transformation, Mutagenesis, Mutant Detection, and Genetic Analysis. Transformation of *Arabidopsis thaliana* Col-0 with *A. tumefaciens* [strain GV3101 (pMP90RK)] carrying plasmid pPCV002-CaMVBT (14) as well as the ethyl methanesulfonate (EMS) mutagenesis of the generated transgenic line *rolB-2* were performed as described (15). For each of 14 independent M2 families, 5000 seedlings were screened for a reverted phenotype 8 days after sowing on solidified Murashige and Skoog medium. The complementation analysis of isolated mutants (Table 1) was done by reciprocal crosses between each pair of mutants and analysis of the seedling phenotype of the F_1 progeny. The frequencies of revertant plants (Table 2) were determined by scoring the plants 8 days after sowing for seedlings showing a completely reverted phenotype as well as 35 days after sowing for plants showing a reverted phenotype of the main shoot.

RNA Analysis. The isolation, separation, transfer, and hybridization of poly(A)$^+$ RNA from plants were described before (15). A 2.2-kb *Eco*RI fragment of plasmid pPCV002-CaMVBT (14) (*rolB* probe), a 1.3-kb *Pvu* II/*Eco*RI fragment of plasmid pPCV002 (16) (*nptII* probe), and a 0.95-kb *Eco*RI fragment of plasmid Pc-UBI 4 (17) (ubiquitin probe) were used for hybridizations.

Isolation of Nuclei and Nuclear Run-Off Transcription Assays. Nuclei were isolated and handled at 4°C. Plant material (5 g) was ground to fine powder in liquid nitrogen, suspended in 40 ml of buffer A (250 mM sucrose/10 mM NaCl/10 mM Mes, pH 6.6/5 mM EDTA/0.15 mM spermine hydrochloride/0.5 mM spermidine phosphate/20 mM 2-mercaptoethanol/0.2 mM phenylmethylsulfonyl fluoride) supplemented with 0.6% Triton X-100 and 2% dextran T40, and briefly homogenized with an Ultra-Turrax (IKA-Works, Cincinnati). After filtration through 50-μm nylon mesh the filtrate was centrifuged for 5 min at 1500 × *g*. The pellet was resuspended in 2 ml of the same buffer and was loaded on a step gradient consisting of consecutive 3.5-ml layers of 2 M sucrose and 80%, 60%, and 40% (vol/vol) Percoll in buffer A, followed by a centrifugation for 15 min at 1000 × *g*. Nuclei were taken from the interface between the sucrose layer and 80% Percoll, diluted 10-fold in buffer B [50 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$/20% (wt/vol) glycerol/10 mM 2-mercaptoethanol], and centrifuged for 5 min at 3000 × *g*. The washing

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Abbreviations: EMS, ethyl methanesulfonate; CaMV, cauliflower mosaic virus.

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Table 1. Crosses and complementation analysis of EMS mutants that cause *rolB* gene silencing in seedlings

Female parent	Male parent						
	M1-1	M6-1	M8-1	M14-1	M15-1	Wild type	*rolB-2*
M1-1	570/575	0/26	0/14	19/19	2/2	0/149	0/137
M6-1	0/38	119/119	29/29	0/24	0/54	0/54	0/44
M8-1	0/26	34/36	98/98	0/12	0/14	0/43	0/45
M14-1	65/66	0/81	0/88	98/98	100/102	0/26	0/54
M15-1	90/90	0/86	0/40	60/63	92/93	0/44	0/66
Wild type	0/94	ND	ND	ND	ND	498/498	0/201
rolB-2	0/77	ND	ND	ND	ND	0/265	0/755

M1-1, M6-1, M8-1, M14-1, and M15-1, mutant designations; ND, not determined. The values given are (number of revertant F_1 progeny in 8-day-old seedlings)/(total number of F_1 progeny).

step was repeated, and nuclei were resuspended in buffer B and stored at −80°C. Aliquots of nuclei were counted in a Neubauer chamber under a fluorescence microscope after staining with 4',6-diamidino-2-phenylindole at 1 μg/ml. To label nascent RNA chains, 3×10^6 nuclei were suspended in 300 μl of buffer B, and 250 units of RNasin, 15 μl of 1 M $(NH_4)_2SO_4$, 10 μl of 0.1 M $MgCl_2$, 4 μl of 0.1 M $MnCl_2$, 11 μl of 20 mM ATP, 11 μl of 20 mM CTP, 11 μl of 20 mM GTP, and 250 μCi of [α-^{32}P]UTP (400 Ci/mmol; 1 Ci = 37 GBq) were added to a final volume of 400 μl and incubated for 30 min at 29°C. The reaction was stopped by addition of 32 μl of tRNA (5 mg/ml), 40 μl of buffer C (200 mM Hepes, pH 7.6/5 mM $MgCl_2$/5 mM $CaCl_2$), and 150 units of RNase-free DNase I followed by an incubation for 30 min at 37°C. Subsequently, 50 μl of buffer D (100 mM Tris·HCl, pH 7.5/50 mM EDTA/10% SDS) and 4 μl of proteinase K (10 mg/ml) were added, and incubation proceeded for another 25 min at room temperature. After phenol/chloroform extraction, RNA was separated from free nucleotides on a NICK column (Pharmacia). Labeled RNA (3×10^6 cpm) was used for hybridization to identical Southern blot filters containing 0.2 μg of DNA of a 0.88-kb *Nru* I/*Eco*RI fragment of plasmid pPCV002-CaMBT (14) (*rolB* probe) and 0.2 μg of DNA of a 0.95-kb *Eco*RI fragment of plasmid Pc-UBI 4 (17) (ubiquitin probe). Hybridizations were performed in 1 M NaCl, 1% SDS, 10% (wt/vol) dextran sulfate, tRNA (100 μg/ml), poly(A) (100 μg/ml), and salmon sperm DNA (100 μg/ml) for 48 h at 65°C. Washing conditions were as described elsewhere (18).

RESULTS

Unstable Expression of the RolB Phenotype in Regenerated Transgenic *Arabidopsis* Plants. By introducing a chimeric construct of the *rolB* gene driven by the cauliflower mosaic virus (CaMV)-35S promoter (14) into *Arabidopsis* via *Agrobacterium*-mediated transformation, we confirmed (13) for *Arabidopsis* that *rolB* transgenic calli were impaired in shoot differentiation. Nevertheless, *rolB* transgenic plants could be regenerated at a very low frequency. Most established *rolB* transgenic lines displayed a variable penetrance and expression of the RolB phenotype among individual progeny plants, suggesting *rolB* gene silencing. Using a different transformation protocol (19), similar results were obtained (B. Masterson and J.S., unpublished results). Hence, the *rolB* gene appears to be a useful tool for monitoring transgene silencing in *Arabidopsis*.

Table 2. Influence of the genetic constitution of *rolB-2* and *egs1-1* on the frequency and developmental timing of *rolB* gene silencing

Genotype		*rolB* gene silencing			
		8-day-old seedlings		35-day-old plants*	
		No. rev./ total	Freq., %	No. rev./ total	Freq., %
rolB-2 / o	+ / +	0 /466	0	13/345	4
rolB-2 / *rolB-2*	+ / +	0 /755	0	276/510	54
rolB-2 / o	*egs1-1* / *egs1-1*	23/247†	9	93/102	91
rolB-2 / *rolB-2*	*egs1-1* / *egs1-1*	570 /575	99	467/470	99

rev., revertants; o, nontransgenic; +, wild-type allele of *egs1* locus; Freq., frequency.
*Phenotypic reversion in the main shoot during rosette formation.
†Appearance of intermediate phenotypes.

Characterization of Meiotically Reversible RolB Silencing in *Arabidopsis*. Expression of the *rolB* gene in *Arabidopsis* results in a variety of phenotypic alterations, including early senescence, pronounced growth retardation, inhibition of hypocotyl and internode elongation, epinastic growth of cotyledons and leaves, altered flower morphology, and sterility (Fig. 1 *A* and *B*, compare r/o;+/+ and o/o;+/+; Fig. 1*D*). Silencing of *rolB* can therefore be visually monitored in somatic tissues by the failure of displaying this RolB phenotype (RolB silencing) (Fig. 1*E*). Among the different *rolB* transgenic lines, line *rolB-2* displayed the strongest RolB phenotype along with an interesting reversible pattern of RolB silencing and was therefore chosen for detailed analysis. The *rolB-2* locus segregates as a single dominant Mendelian factor and consists of three concatenated copies of the transferred T-DNA (arranged in a head-to-head/head-to-tail configuration) at a single genomic integration site (data not shown). Seedlings invariably express a stable RolB phenotype (Fig. 1 *A* and *F*: r/r;+/+ and r/o;+/+; Table 2), while during further development the growing shoot may display RolB silencing. Plants homozygous for the *rolB-2* locus express a strong RolB phenotype, ultimately leading to early senescence. Therefore, RolB silencing in homozygotes is accurately marked by the failure to display early senescence (Fig. 1*C*; sections of the plants expressing *rolB* are senescent while those displaying RolB silencing remain green). The RolB phenotype of hemizygous plants is weaker, resulting in patches of senescent tissue (Fig. 1*D*). Nevertheless, RolB silencing in hemizygotes is reliably marked by the restoration of a normal phenotype and growth rate (Fig. 1*E*; a lateral shoot displaying RolB silencing is marked by an arrow). RolB silencing seems to be triggered by an erratic somatic event that commits the whole meristem to the silenced state (Fig. 1*C*), which is then transmitted stably through mitosis. We have never observed incomplete RolB silencing leading to chimeric tissues or sectoring along the shoot axis (20), nor have we observed somatic reappearance of the RolB phenotype subsequent to RolB silencing. The frequency of RolB silencing is dependent on the genetic state of the *rolB-2* locus. Hemizygous plants display a relatively stable RolB phenotype: only 4% of plants show RolB silencing in the main shoot. In contrast, 54% of homozygous plants displayed RolB silencing in the main shoot (Table 2).

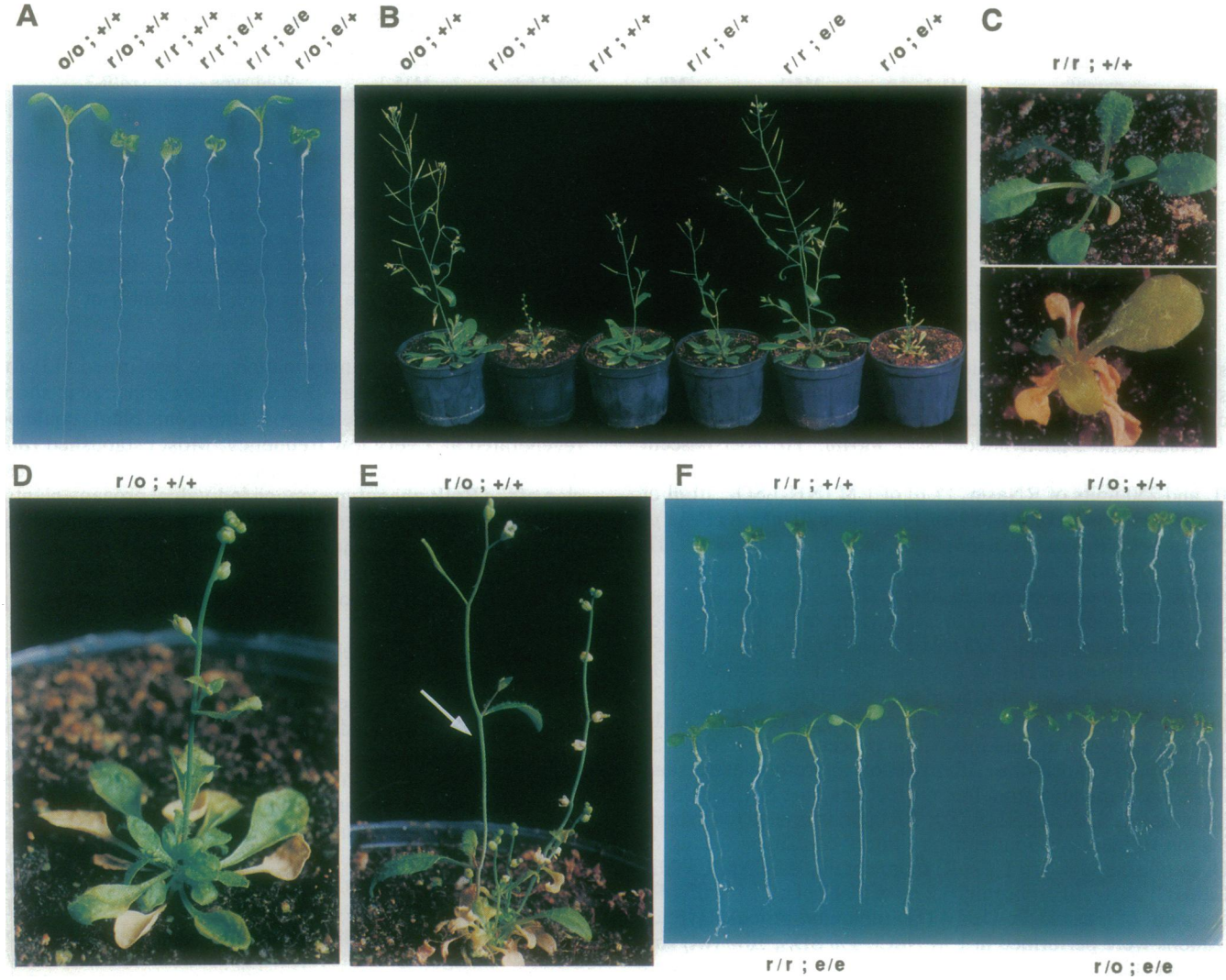

FIG. 1. Phenotypic manifestation of reversible *rolB* gene silencing. Genotypes depicted refer to the *rolB-2* locus [o = wild-type (nontransgenic) constitution; r = *rolB-2* (transgenic) allele] and the *egs1* locus (+ = wild-type allele; e = *egs1-1* mutant allele). Representative phenotypes of corresponding genotypes in 8-day-old seedlings (*A*) and 35-day-old plants (*B*) are shown. (*C*) Twenty-one-day-old plants homozygous for the *rolB-2* locus showing RolB silencing at different stages of rosette formation (sections of the plant expressing *rolB* are senescent while those displaying RolB silencing remain green). Plants hemizygous for the *rolB-2* locus showing a stable RolB phenotype at day 35 (*D*) and late RolB silencing (*E*; arrow points to the revertant lateral shoot) at day 56 are pictured. (*F*) RolB silencing in the *egs1-1* mutant in 8-day-old seedlings.

Viable seeds are formed exclusively after RolB silencing. The resulting seedlings invariably again express the RolB phenotype. This complete resetting of the RolB phenotype seems to occur during, or immediately after, meiosis, since developing seeds were found to express *rolB* only in the embryo and not in the seed coat as analyzed by *in situ* hybridization experiments (H. Meijer and C.D., unpublished results).

Molecular Analysis of Meiotically Reversible RolB Silencing. RolB silencing was found to be correlated with an ≈100-fold reduction of steady-state *rolB* transcripts, whereas steady-state transcript levels of the physically linked *nptII* gene and the endogenous ubiquitin genes were apparently unaffected (Fig. 2, compare r/r;+/+ in *A* and *B*). In contrast, RolB silencing resulted in only an ≈5-fold drop of *rolB* nuclear run-off transcripts (Fig. 3, compare r/r;+/+ in *A* and *B*). Therefore, *rolB* gene silencing seems to result from a predominantly posttranscriptional mechanism.

***Arabidopsis* Mutants That Modify the Frequency and the Timing of Meiotically Reversible RolB Silencing During Plant Development.** Interestingly, no RolB silencing was ever observed during the seedling stage (Table 2). To dissect the process of reversible *rolB* gene silencing, we have screened mutagenized populations for mutants displaying RolB silencing already at the seedling stage. Screening of 70,000 EMS-mutagenized M2 seedlings (obtained from 11,000 M1 plants) homozygous for the *rolB-2* locus resulted in the isolation of five mutants of independent origin, each carrying a single nuclear recessive mutation that is not located within the *rolB-2* locus (see Table 1 for the F_1-progeny analysis of crosses; F_2-segregation data are not shown). Complementation analysis of these mutants (Table 1) allowed definition of two genetic loci: *egs1* for mutants M1-1 (allele *egs1-1*), M14-1 (allele *egs1-2*), and M15-1 (allele *egs1-3*); and *egs2* for mutants M6-1 (allele *egs2-1*) and M8-1 (allele *egs2-2*). The genetic interactions of locus *egs1* (allele *egs1-1*) and the *rolB-2* locus were studied in detail. Plants heterozygous for *egs1-1* were not affected in RolB silencing (Figs. 1 and 2), indicating that the *egs1-1* allele is recessive. In contrast, the *egs1-1* mutant was found to trigger RolB silencing in 99% (compared to 0% for the corresponding wild-type allele) of seedlings homozygous for the *rolB-2* locus (Table 2; Figs. 1*A*, 2*A*, and 3*A*, compare r/r;e/e and r/r;+/+) and 9% (compared to 0% for its wild-type allele) of seedlings hemizygous

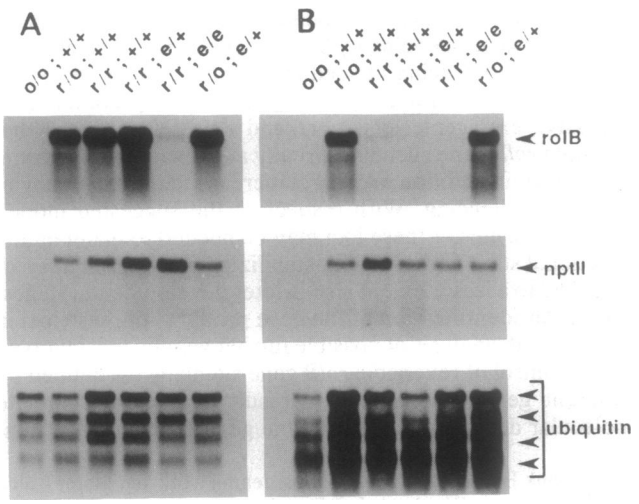

FIG. 2. Effect of reversible *rolB* gene silencing on the steady-state transcript level of the *rolB-2* locus. Genotypes depicted refer to the *rolB-2* locus [o = wild-type (nontransgenic) constitution; r = *rolB-2* (transgenic) allele] and the *egs1* locus (+ = wild-type allele; e = *egs1-1* mutant allele). Eight-day-old seedlings (*A*) and main shoots of 35-day-old plants (*B*) of corresponding genotypes (for representative phenotypes see Fig. 1 *A* and *B*) were subjected to Northern blot analysis of poly(A)$^+$ RNA by serial hybridizations of a single filter with different probes as indicated.

for the *rolB-2* locus (Table 2). The appearance of intermediate phenotypes in seedlings of the *egs1-1* mutant hemizygous for the *rolB-2* locus (Fig. 1*F*; r/o;e/e) may reflect the erratic occurrence of *rolB* gene silencing during embryo and seedling development. In contrast, the much higher frequency of *rolB* gene silencing in seedlings of the *egs1-1* mutant homozygous for the *rolB-2* locus (Table 2, compare r/o;e/e and r/r;e/e) show that at a higher *rolB* gene dosage this process is favored to occur at an early developmental stage, thus leading to phenotypically homogenous seedling populations (Fig. 1*F*, r/r;e/e). During further development (rosette formation), the *egs1-1* mutant displayed RolB silencing in 99% (compared to 54% for the corresponding wild-type allele) of plants homozygous for the *rolB-2* locus (Table 2; Fig. 1*B*, 2*B*, and 3*B*, compare r/r;e/e and r/r;+/+) and in 91% (compared to 4% for the corresponding wild-type allele) of plants hemizygous for the *rolB-2* locus (Table 2).

Two observations suggest that *egs1* is involved in the process of reversible *rolB* gene silencing as described above, rather than in a different and independent gene silencing mechanism at the *rolB-2* locus: (*i*) the *egs1-1* mutant displays RolB silencing more frequently in plants homozygous for the *rolB-2* locus than in hemizygous plants (Table 2), and (*ii*) *rolB* gene silencing observed in the main shoot of unmutagenized plants or in seedlings of the *egs1-1* mutant (both homozygous for the *rolB-2* locus) displays similar molecular characteristics: a dramatic decrease in steady-state *rolB* transcripts in contrast to only a moderate decrease in *rolB* nuclear run-off transcripts (Figs. 2 and 3, compare r/r;e/e in *A* with r/r;+/+ in *B*). Thus, *egs1* seems to be a modifier locus of reversible *rolB* gene silencing. The consequences of the *egs1-1* mutation on this process appear to be twofold: *rolB* gene silencing is triggered at an earlier developmental stage, in the seedling, and the frequency of *rolB* gene silencing is generally increased. Because of this enhancement effect, this modifier locus was named *e*nhancer of *g*ene *s*ilencing (*egs*; see above).

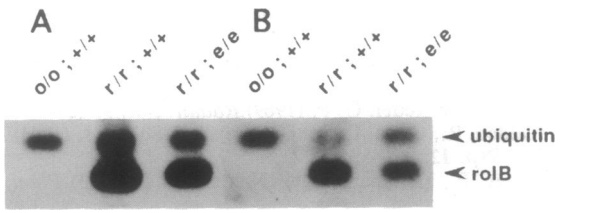

FIG. 3. Effect of reversible *rolB* gene silencing on transcriptional initiation at the *rolB-2* locus. Genotypes depicted refer to the *rolB-2* locus [o = wild-type (nontransgenic) constitution; r = rolB-2 (transgenic) allele] and the *egs1* locus (+ = wild-type allele; e = *egs1-1* mutant allele). Eight-day-old seedlings (*A*) and main shoots of 35-day-old plants (*B*) of corresponding genotypes (for representative phenotypes see Fig. 1 *A* and *B*) were used for nuclear run-off transcription experiments (for experimental details see *Materials and Methods*).

Genetic segregation analysis, Northern blot analysis and nuclear run-off transcription experiments (data not shown) support the notion that the *egs2* locus acts on reversible *rolB* gene silencing in a similar fashion as the *egs1* locus.

DISCUSSION

Transgene silencing in plants has been observed in many different systems. However, the lack of an appropriate model system has hampered progress toward an understanding of the molecular and genetic processes involved. By introducing the *rolB* gene into *Arabidopsis*, we have established a useful model offering a battery of tools to study transgene silencing: (*i*) a selection for transgene silencing because stable *rolB* expression interferes with plant regeneration; (*ii*) a visual assay for transgene silencing based on reversion of the RolB phenotype in transgenic regenerants; and (*iii*) *Arabidopsis* may allow a genetic identification of plant functions involved in transgene silencing. The versatility of this model was used for a detailed analysis of a reversible transgene silencing phenomenon in a particular *rolB* transgenic line. In this line, *rolB* gene expression leads to a pleiotropic phenotype including pronounced growth retardation and early senescence (see Fig. 1*D*). Silencing as seen by the formation of phenotypically normal shoots occurs erratically and is stable through mitosis (see Fig. 1*E*), whereas *rolB* expression is fully restored during or immediately after meiosis. To our knowledge, only two transgene silencing phenomena described in the literature are reminiscent of this pattern of meiotically reversible *rolB* gene silencing: the heterologous expression of a *Nicotiana plumbaginifolia* β-1,3-glucanase gene in *Nicotiana tabaccum* (10) and of a *N. tabaccum* chitinase gene in *Nicotiana sylvestris* (11). Overall similarities between these different systems suggest a similar mechanism, which was analyzed in more detail in this study thanks to the advantages of a visual assay for gene silencing. Whereas in the previous studies gene silencing was correlated with the homozygous state of the transgene (as monitored by Northern blot or Western blot analysis), we could demonstrate that gene silencing also occurs in the hemizygote, albeit with an ≈18-fold lower frequency. Reversible gene silencing, therefore, seems to be dependent on a threshold level of transgene expression, which is more often reached in plants homozygous for the transgene than in hemizygotes.

The molecular characteristics of reversible gene silencing studied in these different systems (refs. 10 and 11 and this study) are consistent with respect to the dramatic decrease of steady-state transgene mRNAs. However, nuclear run-off transcription of the transgene was apparently not affected by gene silencing in ref. 10, whereas we noted a moderate but significant decrease in independent experiments. Although both results argue for a mechanism affecting mRNA stability, the former suggests a purely posttranscriptional mode of action, whereas ours may indicate a mechanism acting at the posttranscriptional level but with an observable effect on *rolB* nuclear run-off transcripts. Alternatively, the moderate decrease of *rolB* nuclear run-off transcription is not a direct

consequence of the gene silencing process but rather reflects the coincident loss of the *rolB* gene product. It was recently demonstrated that transcription of the CaMV-35S promoter is stimulated in *rolB* transgenics (21). Since in our study the *rolB* gene was driven by this promoter, a positive feedback loop might be eliminated by *rolB* gene silencing. Clarification of this aspect awaits further experimental evidence.

In any event, the (predominantly) posttranscriptional nature of reversible gene silencing appears to rule out methylation as a mechanism, since methylation is thought to cause transgene silencing by transcriptional inactivation (22). Consistent with this conclusion, reversible gene silencing was not found to be correlated with the methylation state of several restriction sites analyzed in the transgene locus of ref. 11 or with methylation of a particular *Msp* I/*Hpa* II restriction site in the CaMV-35S promoter (which was shown to be diagnostic for transgene silencing—e.g., in ref. 23) of the chimeric *rolB* gene in this study (data not shown).

The molecular mechanism causing meiotically reversible gene silencing is not known. However, the biochemical switch hypothesis originally formulated by Meins (24) and modified by Jorgensen (3) may serve as a working model for this process since it is in good agreement with the characteristics of reversible gene silencing reported thus far (refs. 10 and 11 and this study). This hypothesis postulates two alternative stable states. A threshold concentration of some product of gene expression (e.g., RNA) would be responsible for the switch. When the threshold is reached, rapid RNA turnover would ensue and gene expression would cease. Here we expand this hypothesis by suggesting a simple molecular mechanism for reversible gene silencing. Our model assumes that the stability of the transgene mRNA (and potentially other mRNA species) is dependent on the specific binding (e.g., via recognition of defined sequences) of a diffusible mRNA-stabilizing gene product and, furthermore, that this mRNA-stabilizing gene product is necessary for its own expression by binding to its own mRNA. If the concentration of the transgene mRNA would reach a certain threshold, it might eventually titrate out the mRNA-stabilizing gene product, resulting in a cessation of gene expression of the locus encoding the mRNA-stabilizing gene product and consequently also of the transgene itself and of other genes, the expression of which is dependent on this mRNA-stabilizing mechanism. Resetting of the mRNA-stabilizing mechanism could then simply result from a transient stabilization of nascent transcripts.

Such a titration model would explain the high basal level of transgene expression found in all cases of reversible gene silencing reported thus far (refs. 10 and 11 and this study). Reversible gene silencing in *Arabidopsis* plants transgenic for the *rolA* gene was indeed only observed in a transgenic line with a very marked RolA phenotype (ref. 15; unpublished results). Our model further predicts that silencing of an ectopically expressed gene should result in cosilencing of the endogenous copy of this gene (because a diffusible mRNA-stabilizing gene product is not expected to discriminate between identical mRNAs). Such an instance was indeed described in ref. 11. Our model also implies that the stability of the *rolB* transcript depends on hitchhiking a mRNA-stabilizing gene product that may control the expression of an as yet unidentified class of genes. *rolB* gene silencing would consequently be expected to result in cosilencing of these genes.

To test our hypothetical model, we analyzed the process of reversible gene silencing by genetic dissection. The absence of *rolB* gene silencing in seedlings allowed us to perform a mutagenic screen for early *rolB* gene silencing events. Two recessive extragenic loci, *egs1* and *egs2*, were found that promote *rolB* gene silencing already at the seedling stage and that cause, in addition, a general increase in the frequency of *rolB* gene silencing. With respect to the suggested model, wild-type alleles of these loci may be needed to maintain the functional state of the mRNA-stabilizing mechanism in seedlings and to a lesser extent also in later developmental stages. Molecular identification of the gene products of these loci in concert with analysis of possible further genetic loci involved in the control of reversible *rolB* gene silencing (as defined by different genetic screens) will result in a more complete molecular description of reversible gene silencing in plants.

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