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Genetics

RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration

(transgene inactivation/DNA–DNA pairing/chromatin structure)

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ABSTRACT **We have previously reported repeat-induced gene silencing (RIGS) in *Arabidopsis*, in which transgene expression may be silenced epigenetically when repeated sequences are present. Among an allelic series of lines comprising a primary transformant and various recombinant progeny carrying different numbers of drug resistance gene copies at the same locus, silencing was found to depend strictly on repeated sequences and to correlate with an absence of steady-state mRNA. We now report characterization, in nuclei isolated from the same transgenic lines, of gene expression by nuclear run-on assay and of chromatin structure by nuclease protection assay. We find that silencing is correlated with absence of run-on transcripts, indicating that expression is silenced at the level of transcription. We find further that silencing is also correlated with increased resistance to both DNase I and micrococcal nuclease, indicating that the silenced state reflects a change in chromatin configuration. We propose that silencing results when a locally paired region of homologous repeated nucleotide sequences is flanked by unpaired heterologous DNA, which leads chromatin to adopt a local configuration that is difficult to transcribe, and possibly akin to heterochromatin.**

In a variety of plant species, repeated transgenic sequences may lead to silenced expression of both transgenes and endogenous genes (1–6). Although molecular mechanisms have yet to be elucidated, present evidence suggests that silencing in different experimental systems may reflect different mechanisms, both transcriptional (7–9) and posttranscriptional (10–16). Proposed explanations have invoked transcriptional inactivation owing to chromatin condensation or *de novo* methylation induced by pairing of DNA (1, 3, 5, 7, 17), posttranscriptional inactivation owing to increased RNA turnover (11, 18, 19), and posttranscriptional inactivation by antisense RNA (14).

Earlier, we reported repeat-induced gene silencing (RIGS) in *Arabidopsis* (20). We used a reporter construct that includes an hygromycin phosphotransferase (*HPT*) gene flanked by two mutant neomycin phosphotransferase II (*npt*) genes carrying different, nonoverlapping deletion alleles, with each gene driven by identical copies of the P35S promoter and nos3′ terminator (Fig. 1). In the primary transformant, abbreviated *3′Δn-H-5′Δn*, recombination events between the directly repeated *npt* genes generated an allelic series of progeny inserts that includes single-copy (*N*) as well as multi-copy (*N-H-5′Δn*, *3′Δn-H-N*, *3′Δn-N*, and *5′Δn-N*) inserts. Some of these insert lines, despite having been selected for drug resistance (Hyg^r or Kan^r), continued to segregate progeny that were drug-sensitive, indicating silencing of expression of both *HPT* and *NPT* (17). Because all recombinant inserts were derived from one primary single-insert transformant, position effects did not apply. Silencing was reversible, and therefore epigenetic. Silencing depended strictly on the presence of repeated sequences and was correlated with absence of steady-state mRNA and with increased methylation. The phenomenon appeared somewhat similar to paramutation in maize (21), and to both repeat-induced point mutation (RIP) (22) and methylation-induced premeiotically (MIP) (23).

Here we report further on gene expression in nuclei isolated from the same transgenic populations described earlier (17). We show that in nuclear run-on mRNA assays silencing is correlated with lack of run-on transcripts, indicating that it is transcriptional. Moreover, in nuclear nuclease protection assays (24) silencing is correlated with increased resistance to digestion. As chromatin is more resistant to digestion when condensed than when extended (25, 26), this result indicates a change in local chromatin configuration. Thus RIGS in *Arabidopsis* is similar to dominant position-effect variegation at the *white* locus in *Drosophila*, where genetic evidence suggests that somatic pairing of closely linked repeats can cause local heterochromatin formation and transgene silencing (27).

MATERIALS AND METHODS

Plant Lines and Growth Conditions. As described in previous work (17), all silenced and nonsilenced sublines used in this study are derived from a single primary callus transformed to Hyg^r with the test construct *3′Δnpt-HPT-5′Δnpt* (Hyg^r Kan^s) and subsequently shown to carry a single insert. Primary (i.e., nonrecombinant Hyg^r Kan^s) sublines derived by callus subdivision showed different degrees of *HPT* silencing, as did recombinant sublines derived by selection for Kan^r and subsequently shown to carry one copy of *NPT* with or without additional copies of *npt* and in some cases *HPT* as well. As the original transformant was hemizygous, recombinant sublines could be either heterozygous (R/P, where R indicates recombinant and P indicates primary insert), homozygous (R/R), or hemizygous (R/−).

As described extensively earlier (17), whereas the silenced primary subline described here (1h11; see below) was silenced completely, each of the various silenced recombinant sublines described here (see below) segregated a progeny population that showed a particular characteristic distribution of silencing extent. Consequently, it is important to note that each of the populations described in the present work was grown from the very same pool of seeds that was used for the phenotypic characterization of that population reported earlier (17). As each silenced population in fact included a small number of individuals unsilenced to varying extents, the molecular correlates reported below are minimal estimates.

The lines used were as follows: Be-0, wild-type ecotype Bensheim (−/−); 1p, primary (P/P) Hyg^r (unsilenced) T2 generation; 1h11, primary (P/P) Hyg^s (silenced) T3 generation; NR6.2c1, recombinant single-copy *NPT* (R/R) Kan^r (unsilenced) T7 generation; NR8.1, recombinant multicopy *3′Δnpt-HPT-NPT* (R/P) Kan^r (unsilenced) T5 generation; NR10.2b, recombinant multicopy *3′Δnpt-HPT-NPT* (R/P) Kan^s (silenced) T6 generation; NR3.1c, recombinant multicopy *NPT-HPT-5′Δnpt* (R/R or R/P) Kan^r (unsilenced) T6

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Abbreviation: RIGS, repeat-induced gene silencing.

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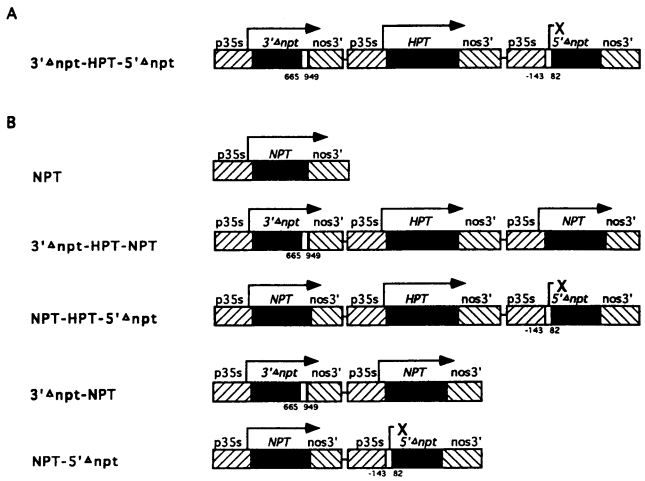

FIG. 1. Structure of primary transgene insert (*A*) and derived recombinant inserts (*B*). In the primary insert, two mutant *npt* alleles in the same orientation flank an active *HPT* gene. The various recombinant inserts have resulted from independent gene conversion and/or crossover events (20). 3′Δ*npt* is a 284-bp *Nae*I deletion spanning bp 665–949 in *NPT* (white box); 5′Δ*npt* is a 225-bp *Bal*31 deletion spanning bp −143 in *P35S* to bp 82 in *NPT* (white box); *P35S* is the cauliflower mosaic virus promoter; and *nos3′* is the nopaline synthase terminator. Arrows indicate transcription, and truncated arrows indicate lack of transcription.

generation; NR3.1c3, recombinant multi-copy *NPT-HPT-5′Δnpt* (R/R or R/−) Kan^s (silenced) T7 generation; NR9.1d, recombinant multicopy 3′Δ*npt-NPT* (R/R or R/P) Kan^r (unsilenced) T6 generation; NR15, recombinant multicopy 3′Δ*npt-NPT* (R/P) Kan^s (silenced) T7 generation.

For tests of drug response, seeds were surface-sterilized and plated on MS medium containing 1% sucrose, 0.5% agar, and either 50 μg/ml kanamycin or 20 μg/ml hygromycin. Plates were incubated at 20°C with 16-hr day/8-hr night cycles. Resistant and sensitive seedlings were scored after 7 days and transferred to soil.

Isolation of Nuclei. Plants were grown for 3 weeks. Ten grams of Aerial tissue (about 60 plants) was ground to fine powder in liquid nitrogen with a mortar and pestle, the powder was resuspended in 120 ml of homogenization buffer (HB; 0.4 M sucrose/0.6% Triton X-100/5% dextran T 40/2.5% Ficoll/25 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/0.15 mM spermine/0.5 mM spermidine/0.2 mM phenylmethylsulfonyl fluoride/10 mM 2-mercaptoethanol). The suspension was filtered through two layers of Miracloth (Calbiochem) and then through two layers of nylon mesh (80 and 50 μm). Crude nuclear pellets were harvested by centrifugation at 4000 × *g* for 5 min (4°C).

Nuclear pellets were resuspended in 20 ml HB (without spermine and spermidine) and further purified (4 × 5 ml) by centrifugation in a step gradient of 5-ml layers of 2 M sucrose, and 80%, 60%, and 40% Percoll in HB (3000 × *g*, 30 min). Nuclei were taken from the interface between the sucrose layer and 80% Percoll, diluted 10-fold in wash buffer (50 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$/20% glycerol/0.25% Triton X-100/10 mM 2-mercaptoethanol), and washed twice with the same buffer by centrifugation at 4000 × *g* for 5 min. Finally, the pelleted nuclei were resuspended in 800 μl storage buffer (50 mM Tris·HCl, pH 8/5 mM $MgCl_2$/25% glycerol/10 mM 2-mercaptoethanol) and stored at −80°C.

To determine the yield of nuclei, 10 μl nuclear suspension was mixed with 10 μl of 2 μg/ml 4′,6-diamidino-2-phenylindole in storage buffer. After 2-min incubation on ice, nuclei were counted in a Neubauer chamber under a fluorescence microscope.

Nuclear Run-On Assay. *In vitro* RNA-synthesizing reactions of isolated nuclei were carried out basically as described by Dehio and Schell (18). Three hundred microliters of nuclei in storage buffer (3.5 × 10^6), 100 μl of buffer A [160 mM $(NH_4)_2SO_4$/10 mM M_gCl_2/4 mM M_nCl_2/2 mM each CTP, GTP, and ATP), 300 units of RNasin, and 100 μCi of [α-^{32}P]UTP (800 Ci/mmol; 1 Ci = 37 GBq; Amersham) were mixed. After incubation at 28°C for 30 min, 250 μg/ml tRNA, 20 mM Hepes (pH 7.6), 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 150 units RNase-free DNase I (Sigma) were added to the reaction mixture. After an additional 20 min at 37°C, 10 mM Tris·HCl (pH 7.5), 5 mM EDTA, 1% SDS, and 100 μg/ml proteinase K were added, and incubated for 30 min at room temperature. Nucleic acids were purified by phenol-chloroform (1:1) extraction and recovered by ethanol precipitation. The pellet was resuspended in 50 μl TE (pH 7.5) and passed through a NICK column (Pharmacia). Finally, the labeled RNA was precipitated with ethanol and the pellet was resuspended in 20 μl H_2O.

For Southern blotting experiments, DNA fragments (0.25 μg) containing gene-specific sequences was denatured and bound to nylon membrane (Hybond-N$^+$; Amersham) and hybridized with labeled RNA (2 × 10^6 cpm). Hybridizations were performed in 5× SSPE (standard saline phosphate/EDTA), 5× Denhardt's solution, 0.5% SDS, 100 μg/ml tRNA, 100 μg/ml poly(A), and 100 μg/ml salmon sperm DNA for 48 hr at 65°C. The blots were washed twice with 1× SSPE/0.5% SDS and once with 0.2× SSPE/0.5% SDS at 65°C for 30 min.

DNase I Digestion. Two hundred fifty microliters of nuclear suspension (≈3 × 10^6 nuclei) were treated with 0.5 unit/ml DNase I (Worthington) at 25°C for 0, 1, 2, 5, and 10 min. The reaction was stopped with equal volume of stop buffer (100 mM Tris·HCl, pH 7.5/600 mM NaCl/2% SDS/100 mM EDTA/1 mg of proteinase K per ml), and incubated at 50°C for 4 hr. DNA was extracted twice with phenol/chloroform and precipitated with ethanol in the presence of 0.2 M NaCl, and the pellets were resuspended in 200 μl TE. After the addition of 2 μl 10 mg/ml RNase A, the reaction was incubated at 37°C for 1 hr. The DNA was phenol/chloroform extracted and precipitated with ethanol, and the pellet was resuspended in 30 μl H_2O. The DNA was further digested with *Hin*dIII or *Bam*HI (5 units/μg DNA) for 16 hr, and then extracted with phenol/chloroform and precipitated with ethanol. The recovered DNA (20 μg) was analyzed by electrophoresis on 1.0% agarose gels and transferred to nylon membrane (Hybond-N$^+$; Amersham). The blots were hybridized with ^{32}P-labeled probes and autoradiographed with intensifying screens for up to 6 days. Blots hybridized with multiple probes were stripped with boiling 0.5% SDS between successive hybridizations.

Micrococcal Nuclease Digestion. Two hundred fifty microliters of nuclear suspension (≈3 × 10^6 nuclei), 1 μl 250 mM $CaCl_2$, and 25 units/ml micrococcal nuclease (Worthington) were mixed and incubated at 37°C for 0, 1, 2, 5, 10, and 20 min. Digestion was stopped with equal volume of stop buffer, DNA was prepared by phenol/chloroform extraction and ethanol precipitation, and Southern analysis done as described above.

RESULTS

Nuclear Run-On Analysis. In this experimental system, silencing is observed in sibling sublines of the primary transformant line and also in recombinant progeny lines that carry repeated sequences (17). Silencing is correlated with absence of steady-state mRNA (17). To determine whether mRNA is blocked at the transcriptional or the posttranscriptional level, run-on assays were performed with nuclei isolated from silenced and unsilenced primary sublines, and from isogenic silenced and unsilenced recombinant progeny lines (Fig. 2). *NPT* and/or *HPT* run-on transcripts were found in nuclei from an unsilenced primary transformant subline (1p), and from unsilenced recombinant progeny lines containing one copy (6.2c1) or more than one copy (NR8.1, NR3.1c, and NR9.1d) of the transgenes. By contrast, no run-on transcripts were found in nuclei from either a silenced primary subline (1.h11) or silenced multicopy progeny recombinant lines (NR10.2b, NR3.1c3, and NR15). As control, internal *ABT* (*Arabidopsis* β-tubulin) transcripts were found in all cases,

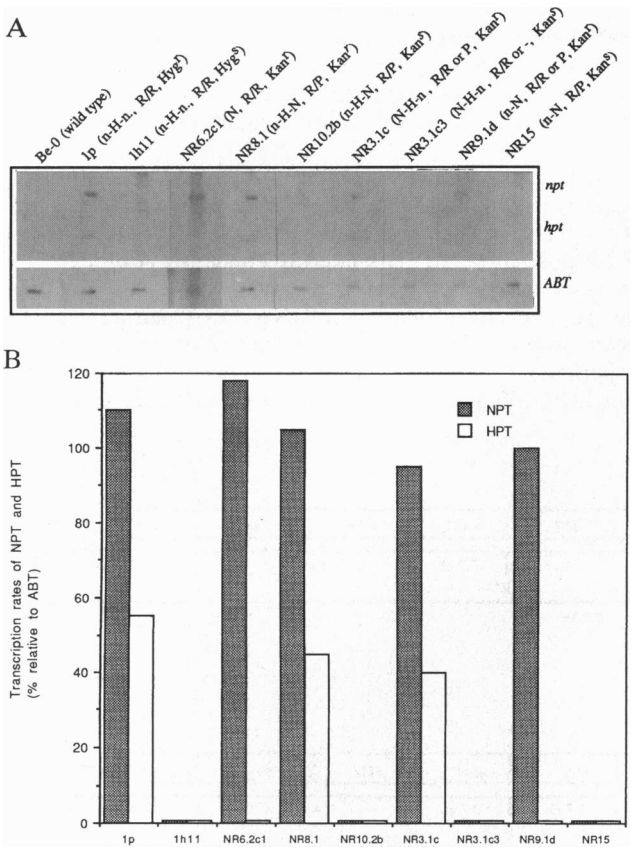

FIG. 2. Nuclear run-on transcription analysis of primary insert and recombinant insert sublines. (*A*) Hybridization of ^{32}P-labeled run-on transcripts to gel separated gene specific DNA fragments. (*B*) Quantitation of hybridized transcriptions. *NPT* and *HPT* transcription rates (percentage of β-tubulin *ABT*) are shown. *npt*, 2.0-kb *Bgl*II–*Cla*I fragment of pFA39 (20); *hpt*, 1.1-kb *Bam*HI coding region fragment of P35S:Hyg (17); *ABT*, 1.0-kb *Kpn*I–*Bam*HI fragment of pABT (28). Be-0, wild-type control (nontransgenic); 1p, primary insert Hyg^r T2 line (unsilenced); 1.h11, primary insert Hyg^s T3 line (silenced); NR6.2c1, Kan^r single-gene *NPT* (R/R) T7 line (unsilenced); NR8.1, Kan^r multi-gene 3′Δ*npt-HPT-NPT* (R/P) T5 line (unsilenced); NR10.2b, Kan^s 3′Δ*npt-HPT-NPT* (R/P) T6 line (silenced); NR3.1c, Kan^r *NPT-HPT-5′Δnpt* (R/R or R/P) T6 line (unsilenced); NR3.1c3, Kan^s *NPT-HPT-5′Δnpt* (R/R or R/−) T7 line (silenced); NR9.1d, Kan^r 3′Δ*npt-NPT* (R/R or R/P) T6 line (unsilenced); NR15, Kan^s 3′Δ*npt-NPT* (R/P) T7 line (silenced). R, recombinant insert chromosome; P, primary insert chromosome; -, chromosome with no insert; s, sensitive; r, resistant.

silencing of expression induced by repeated sequences thus reflects a block in mRNA transcription.

DNase I Digestion. Eukaryotic transcription may be blocked by condensation of chromatin (29, 30). To determine if the silencing observed in our *Arabidopsis* lines is correlated with changes in local chromatin structure, we assayed sensitivity of the transgenes in nuclei isolated from several silenced and unsilenced primary sublines and recombinant lines to digestion with DNase I. Genetic maps of these lines are shown in Fig. 1, and relevant restriction sites in Fig. 3*A*.

When DNase I digested DNA was further digested with *Hin*dIII (Fig. 3*B*), primary sublines 1p (unsilenced) and 1.h11 (silenced) showed bands at 8.0 kb (*P35S-5′Δnpt-nos3′* plus downstream host sequence), 4.2 kb (*P35S-3′Δnpt-nos3′* plus upstream host sequence), and 3.0 kb (*P35S-HPT-nos3′*). Single-copy recombinant line 6.2c1 (unsilenced) showed a single band at 12.2 kb (*P35S-NPT-nos3′* plus upstream and downstream host sequences). For unsilenced primary subline 1p and recombinant line 6.2c1, all fragments were extensively digested after 2-min DNase I treatment. In contrast, for silenced primary subline 1.h11 the 4.2-kb and 3.0-kb fragments were still detectable even after 10-min incubation (Fig. 3*B*). The control 6.0-kb fragment of the (unsilenced) endogenous *ABT* was equally sensitive to DNase I digestion in both silenced and unsilenced lines.

When DNase I-digested DNA was further digested with *Bam*HI (Fig. 3 *C* and *D*), primary sublines 1p (silenced) and 1.h11 (unsilenced) showed bands at 9.0 kb (*P35S-3′Δnpt* plus upstream host sequence), 4.8 kb (*nos3′* plus downstream host sequence), 2.4 kb (*P35S-5′Δnpt*), 2.3 kb (*nos3′-P35S*), 1.1 kb (*HPT*), and 0.7 kb (*nos3′*). Single-copy recombinant line 6.2c1 (unsilenced) showed bands at 9.2 kb (*P35S-NPT* plus upstream) and 4.8 kb (*nos3′* plus downstream host sequence). It is understandable that the 1.1-kb *hpt* band was not observed in 6.2c1 line, since this single copy *NPT* recombinant line does not contain *HPT* gene (homozygous, Fig. 4 *Top*). Recombinant progeny lines NR3.1c (unsilenced) and NR3.1c3 (silenced) containing more than one copy of the transgenes showed bands at 9.2 kb (*P35S-NPT* plus upstream host sequence), 4.8 kb (*nos3′* plus downstream host sequence), 2.4 kb (*P35S-5′Δnpt*), 2.3 kb (*nos3′-P35S*), 1.1 kb (*HPT*), and 0.7 kb (*nos3′*). For unsilenced primary subline 1p, and recombinant lines 6.2c1 and 3.1c, all fragments were extensively digested after 2-min DNase I treatment. For silenced primary subline 1.h11 and recombinant line 3.1c3, however, the 4.8-, 2.4-, 2.3-, and 0.7-kb fragments were still detectable. The control 7.8-kb fragment of the (unsilenced) endogenous *ABT* was equally sensitive to DNase I digestion in both silenced and unsilenced lines (Fig. 3 *C* and *D*).

At least one to three hypersensitive sites were detectable with the *NPT* probe in unsilenced primary subline 1p and recombinant lines 6.2c1 or 3.1c, but not in silenced primary subline 1.h11 or recombinant line 3.1c3 (Fig. 3 *C* and *D*). It is possible that those hypersensitive sites may localize very close to the *Hin*dIII cut site (s), or in the 5′end region of the 9.0-kb (or 9.2-kb) *Bam*HI fragment, which is out of the range of 4.2-kb (or 4.4-kb) *Hin*dIII fragment (Fig. 3 *C* and *D*). The similarity of restriction fragment sizes for the different transgenes (Fig. 3*A*) precluded more extensive analysis of hypersensitive sites.

Fig. 3 also shows that even in silenced lines, fragments including host sequences upstream and downstream of the transgenic insert were more sensitive to DNase I digestion than internal fragments. One possibility is that the flanking regions happen to be in a more extended chromatin state. Another possibility, supported by the rapid disappearance of larger fragments (e.g., 9.0 kb *P35S-3′Δnpt*, 9.2 kb *P35S-NPT*, 7.8 kb *ABT*), is that high molecular weight bands are more sensitive to digestion because they present larger targets for the enzyme (31). Weak bands observed at zero time (Fig. 3 *C* and *D*) are presumed to result from partial digestion by endogenous nucleases (32).

Micrococcal Nuclease Digestion. Similar results were obtained with micrococcal nuclease digestion (Fig. 4). For silenced primary subline 1.h11, the silenced *NPT* gene was digested more slowly than the (unsilenced) control *ABT* gene. In contrast, for unsilenced primary subline 1p and single-copy recombinant line 6.2c1 the *NPT* and *ABT* genes were digested at similar rates (best seen at 2-min digestion, Fig. 4). Similar results were observed with specific *P35S and nos3′* probes rather than *NPT* (data not shown).

DISCUSSION

RIGS in these transgenic *Arabidopsis* lines has been described at the level of phenotype, and correlated with both absence of steady-state mRNA and increased methylation (17). Results reported here extend and amplify those observations. As noted above (*Materials and Methods*), each of the subline populations referred to here as silenced necessarily included the small and characteristic proportion of individuals unsilenced to varying extents, described extensively earlier (17). Consequently, the molecular correlates of silencing reported here may be considered minimal estimates.

In principle, absence of steady-state mRNA could reflect either lack of transcription or posttranscriptional degradation. These two alternatives can be distinguished by assay of

run-on transcription in isolated nuclei. Fig. 2 clearly shows the presence of run-on transcripts in unsilenced lines, but the absence of such transcripts in silenced lines. This suggests that in the silenced lines a functional transcription complex is not present on transgenic insert DNA, and that in these lines silencing is imposed at the level of transcription. Thus this evidence clearly distinguishes the present system from other plant systems in which silencing appears to be post-transcriptional (10–18).

Eukaryotic DNA *in vivo* is packaged in chromatin, which when transcribed is extended and when untranscribed is condensed (29, 30). As the extended state is more accessible than the condensed state to digestion by nucleases, these two states can be distinguished by assay of nuclease protection (33). Figs. 3 and 4 show clearly that transgenic insert DNA is more readily digested by both DNase I and micrococcal nuclease in unsilenced than silenced lines. This indicates that in these lines silencing is correlated with a change in chromatin configuration. Whether silencing is the consequence of this chromatin change, or alternatively its cause, remains unresolved by these data.

Silencing in this system depends strictly on repeated DNA sequences, although it is not an obligatory consequence of their presence (17). It follows that repeated sequences may predispose chromatin ultimately to condense. Although there is no obvious mechanism for that, it seems reasonable to invoke some form of somatic DNA–DNA pairing. This has been proposed for silencing of expression in a variety of experimental systems not only in plants (1, 7, 17) but also in fungi (22, 23), yeast (29, 34) and *Drosophila* (27). In many of

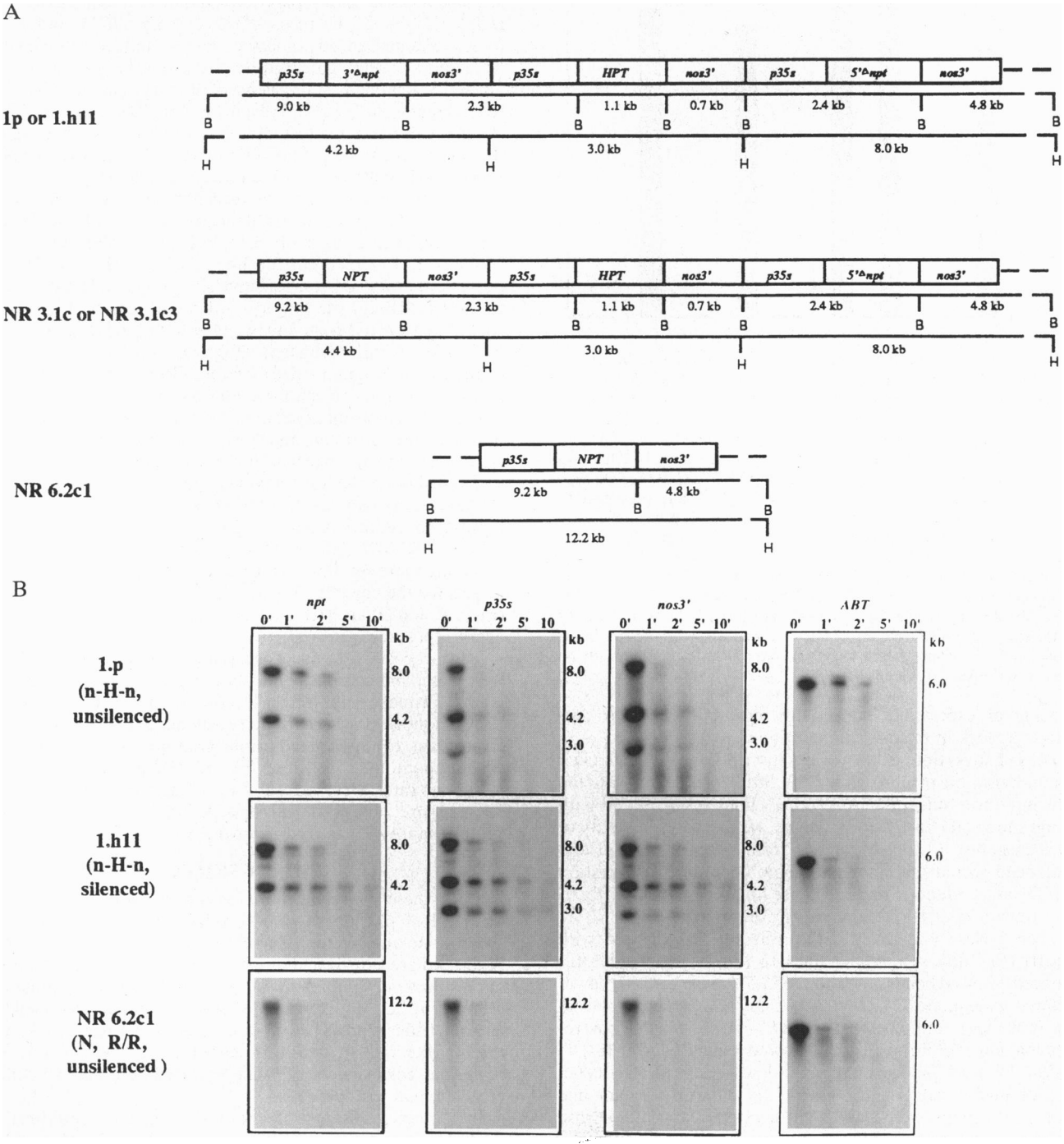

FIG. 3. (*Figure continues on opposite page.*)

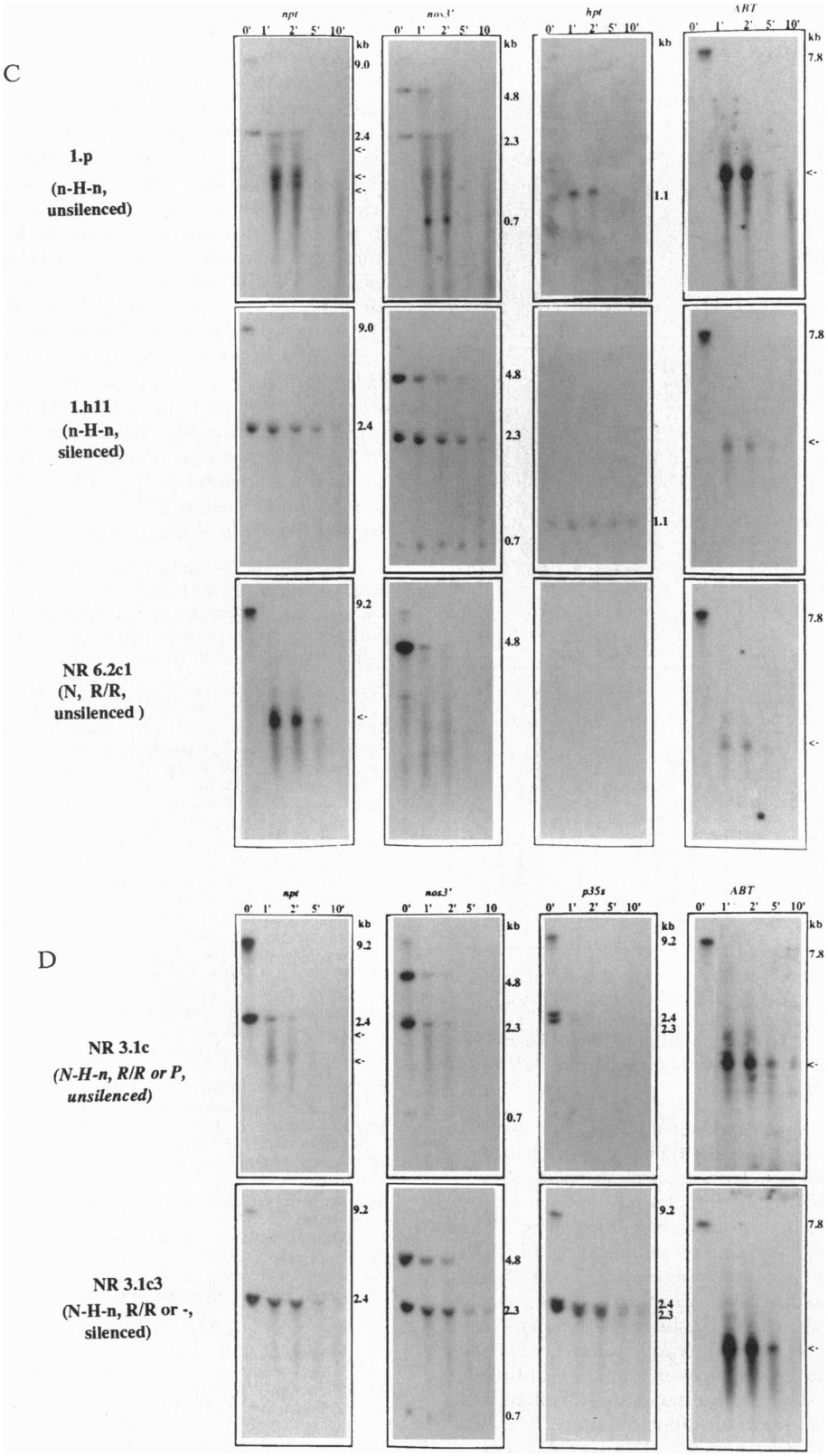

FIG. 3. Digestion of transgene chromatin in isolated nuclei by DNase I. (*A*) Simplified map of *Bam*HI and *Hin*dIII restriction sites. (*B*) *Hin*dIII digest. (*C* and *D*) *Bam*HI digests. *npt* probe is the 355-bp *Pst*I–*Sph*I fragment of pFA39 (20); *P35S* probe, the 0.5-kb *Eco*RI–*Sca*I fragment of *P35S* (17); *nos3'* probe, the 1.1-kb *Hin*dIII–*Bam*HI fragment of *nos3'* (17); *hpt* probe, the 1.1-kb *Bam*HI coding region fragment of P35S:Hyg (17); *ABT* probe, the 1.00kb *Kpn*I–*Bam*HI fragment of pABT (28). 1p, primary insert Hyg^r T2 line (unsilenced); 1.h11, primary insert Hyg^s T3 line (silenced); NR6.2c1, Kan^r single-gene *NPT* (R/R) T7 line (unsilenced); NR3.1c, Kan^r *NPT-HPT-5'Δnpt* (R/R or R/P) T6 line (unsilenced); NR3.1c3, Kan^s *NPT-HPT-5'Δnpt* (R/R or R/−) T7 line (silenced). Arrows at right indicate hypersensitive sites.

these systems including our own increased methylation is observed as well (3–6, 17), although whether methylation is the cause or the consequence of change in chromatin configuration, or indeed of silencing, is not at all clear.

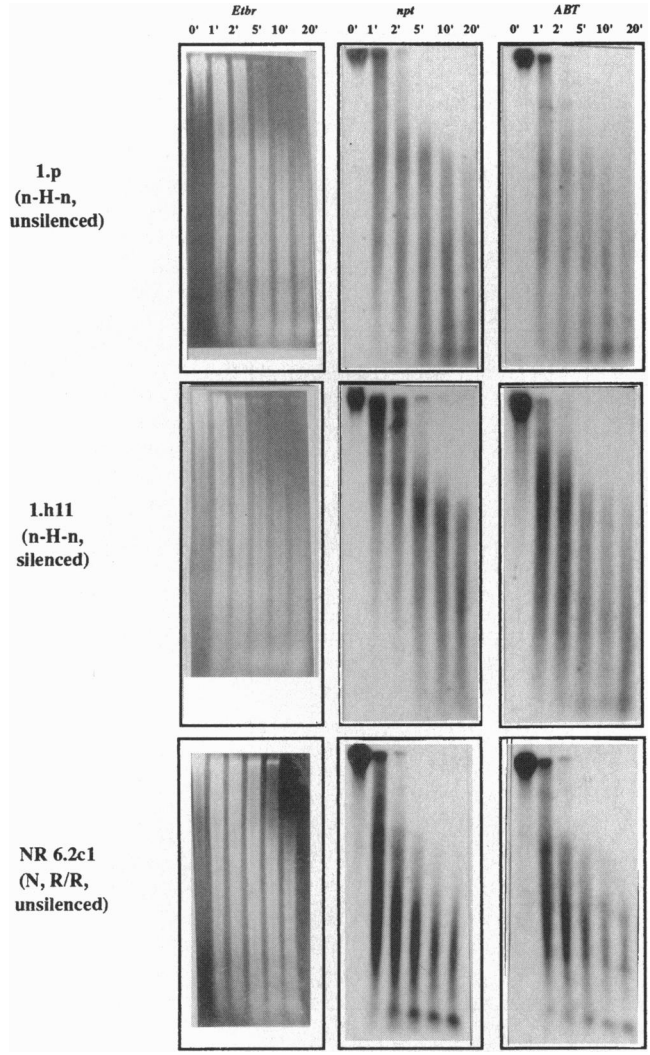

FIG. 4. Micrococcal nuclease digestion of transgene chromatin in isolated nuclei. *npt* probe, 355-bp *Pst*I–*Sph*I fragment of pFA39 (20); *ABT* probe, 1.0-kb *Kpn*I–*Bam*HI fragment of pABT (28). (*Top*) Primary insert Hyg^r T2 line 1p (unsilenced). (*Middle*) Primary insert Hyg^s T3 line 1.h11 (silenced). (*Bottom*) Kan^r single-gene *NPT* (R/R) T7 line 6.2c1 (unsilenced).

Silencing reflects the transition of an actively transcribed gene into a nontranscribed state. Although the mechanism of this phenomenon is still not clear, the present results serve to focus attention on DNA–DNA interactions, at least here. In our system, repeated sequences involved in silencing may be located in *trans*, that is, on different chromosomes (17). Given that the plant is diploid, it is not apparent why those repeated sequences should be silenced when ordinary allelic pairs are not. In principle the plant might distinguish transgenic from endogenous sequences and be able to silence only the former, but there is no obvious molecular basis for such a distinction, and moreover in other systems a transgenic and an endogenous pair may be silenced in *trans* ("cosuppression") (1).

For endogenous allelic pairs, however, DNA homology extends the length of the chromosome. By contrast, for both cosuppression as well as the *trans* silencing in our system (17), DNA flanking the repeated sequences on either side is heterologous. We propose, therefore, that for silencing to result, not only must the DNA sequences to be silenced be able to pair, but in addition, the DNA flanking that paired region must be heterologous. We imagine that such a configuration renders the paired region inaccessible to RNA polymerase, and also to proteins that keep chromatin extended and possibly methylated as well (35). We would then conjecture that additional mechanisms have evolved to allow certain endogenous repeated sequences (e.g., gene families) to remain unsilenced nevertheless.

RIGS in *Arabidopsis* thus resembles dominant position-effect variegation at the *white* locus in *Drosophila*, in depending on pairing of repeated sequences, involving local change in chromatin configuration, and leading to transgene silencing (27). In *Drosophila* it has been suggested that this effect is similar to that resulting from packaging of DNA into heterochromatin (36), and in particular, that somatic pairing can lead to sequestration of normally euchromatic genes into a heterochromatic compartment (37). Our results are quite consistent with that suggestion, and raise the possibility that RIGS in *Arabidopsis* may be functionally related to the formation of heterochromatin. The role of heterochromatin is not understood, however, and thus this possibility is difficult to evaluate as yet.

In summary, we have shown that RIGS in *Arabidopsis* is transcriptional and involves change in chromatin configuration. We propose that gene expression is silenced owing to a chromatin configuration that is brought about when a region of paired homologous DNA is flanked by unpaired heterologous DNA. Tests of this proposal are now in progress.

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