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Genetics

Gene dosage and stochastic effects determine the severity and direction of uniparental ribosomal RNA gene silencing (nucleolar dominance) in *Arabidopsis* allopolyploids

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ABSTRACT **Nucleolar dominance is an epigenetic phenomenon in which one parental set of ribosomal RNA (rRNA) genes is silenced in an interspecific hybrid. In natural *Arabidopsis suecica*, an allotetraploid (amphidiploid) hybrid of *Arabidopsis thaliana* and *Cardaminopsis arenosa*, the *A. thaliana* rRNA genes are repressed. Interestingly, *A. thaliana* rRNA gene silencing is variable in synthetic *Arabidopsis suecica* F_1 hybrids. Two generations are needed for *A. thaliana* rRNA genes to be silenced in all lines, revealing a species-biased direction but stochastic onset to nucleolar dominance. Backcrossing synthetic *A. suecica* to tetraploid *A. thaliana* yielded progeny with active *A. thaliana* rRNA genes and, in some cases, silenced *C. arenosa* rRNA genes, showing that the direction of dominance can be switched. The hypothesis that naturally dominant rRNA genes have a superior binding affinity for a limiting transcription factor is inconsistent with dominance switching. Inactivation of a species-specific transcription factor is argued against by showing that *A. thaliana* and *C. arenosa* rRNA genes can be expressed transiently in the other species. Transfected *A. thaliana* genes are also active in *A. suecica* protoplasts in which chromosomal *A. thaliana* genes are repressed. Collectively, these data suggest that nucleolar dominance is a chromosomal phenomenon that results in coordinate or cooperative silencing of rRNA genes.**

Nucleolar dominance was among the first recognized epigenetic phenomena, discovered in interspecific hybrids in the plant genus *Crepis*. Navashin noted secondary constrictions at metaphase on D chromosomes inherited from one *Crepis* species, but not on the D chromosome from the other species (1, 2). Navashin's contemporary, McClintock, showed that these secondary constrictions are sites where nucleoli were organized in the preceding interphase (3). Decades later, nucleolus organizer regions (NORs) were identified as loci where genes encoding the precursor of the 18S, 5.8S, and 25–28S ribosomal RNAs are tandemly arrayed (4–7). Thus, nucleolar dominance was shown ultimately to result from uniparental rRNA gene expression (8).

Nucleolar dominance occurs throughout the plant and animal kingdoms (9, 10) via mechanisms that remain unclear. Navashin noted that dominant NOR-bearing chromosomes could be contributed through the pollen or egg (2), ruling out maternal or paternal effects. A dominant D chromosome could also be contributed as part of an incomplete chromosome set. In *Drosophila melanogaster* × *Drosophila simulans* hybrids, the *D. melanogaster* NORs on the X and Y chromosomes are dominant over the single NOR on the *D. simulans* X (11). In hybrid XO males lacking a *D. melanogaster* sex chromosome, but containing all autosomes, the *D. simulans* NOR is active. These results indicate that NOR-bearing chromosomes must be present for nucleolar dominance to occur. Evidence from *Xenopus* suggested that the rRNA genes themselves might be responsible. In hybrids of *X. laevis* and *X. borealis*, only *X. laevis* rRNA genes are expressed in early development (12, 13). Using *X. laevis* and *X. borealis* rRNA minigenes coinjected into oocytes, Reeder and Roan found that sequences in the *X. laevis* intergenic spacer conferred transcriptional dominance (14). They hypothesized that *X. laevis* rRNA genes compete better than *X. borealis* genes for one or more limiting transcription factors because of an increase in enhancer number or strength (14). A similar hypothesis was proposed for nucleolar dominance in wheat (15, 16).

Nucleolar dominance has been thought to be independent of rRNA gene dosage or ploidy. Navashin observed dominant D chromosomes suppressing secondary constrictions on as many as three underdominant D chromosomes (in a 1:3 allotetraploid). Likewise, dominant *Brassica* rRNA genes can be outnumbered in a 4:2 allohexaploid (17) and the dominant NOR in hexaploid wheat has only half as many rRNA genes as the second most active NOR (18, 19). The favored, although unproved, explanation has been that rRNA genes are present in excess over transcription factors such that dominant genes titrate these factors even when outnumbered.

We report the occurrence and molecular analysis of nucleolar dominance in *Arabidopsis suecica*, an allotetraploid of *Arabidopsis thaliana* and *Cardaminopsis arenosa* (20). Surprisingly, *A. thaliana* rRNA gene silencing in newly formed *A. suecica* hybrids is highly variable, with two generations needed to establish dominance in some lines. Also, unexpectedly, the direction of dominance can be switched (*C. arenosa* genes silenced) in 3:1 *A. thaliana* to *C. arenosa* allotetraploids, an observation that challenges the hypothesis that dominant genes have inherently higher binding affinities for transcription factors. The possibility that uniparental rRNA gene expression results from silencing a species-specific transcription factor is argued against by transient expression experiments. These results suggest that nucleolar dominance is a chromosomal phenomenon that is enforced independent of transcription factor availability.

MATERIALS AND METHODS

Plant Material. *A. suecica* race 90.10.085 was provided by Dr. Steven O'Kane (University of Northern Iowa, Cedar Falls, IA). Tetraploid *A. thaliana* Landsberg *erecta* plants were regenerated from cultured roots (21) treated with 0.5% (vol/vol) colchicine. A single *C. arenosa* plant from race 9509

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Abbreviations: NOR, nucleolus organizer region; rRNA, ribosomal RNA; SAS, synthetic *A. suecica.*

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(designated Care-1 in the Comai laboratory) was the pollen donor onto emasculated flowers of tetraploid *A. thaliana* to create synthetic allotetraploid *A. suecica*-like hybrids [designated synthetic *A. suecica* (SAS)]. Four self-fertile SAS F_1 plants were obtained: SAS 1-4 (original strain designations are 605A, 605B, 49-2B, and 49-2A). F_1 plants were self-pollinated to produce F_2 seeds. SAS-2 was the female parent for backcrosses to tetraploid *A. thaliana* or *C. arenosa*. Chromosome analyses confirmed that SAS plants were allotetraploid; biparental inheritance of multiple molecular markers was also confirmed (L.C. *et al.*, unpublished work).

Nucleic Acid Isolation. Nucleic acids were generally purified from pooled tissues of 5–10 plants. For SAS F_1 and F_2 plants and backcross progeny, leaves from single plants were used. Tissues frozen in liquid nitrogen were ground to a powder, then mixed with three volumes (wt/vol) of extraction buffer (250 mM Tris·HCl, pH 8.5/375 mM NaCl/25 mM EDTA, pH 8.0/1% SDS/1% β-mercaptoethanol/0.5 mg/ml heparin). The homogenate was extracted twice with phenol/chloroform, and total nucleic acids were ethanol precipitated (22). Following centrifugation, pellets were resuspended in diethylpyrocarbonate-treated sterile water and total RNA was precipitated with 3 M LiCl. Genomic DNA in the supernatant was recovered by ethanol precipitation.

PCR Amplification. PCR was used to amplify rRNA gene sequences from −265 (relative to the *A. thaliana* transcription start site, +1) to the 18S rRNA coding region using genomic DNA from *A. thaliana*, *A. suecica,* and *C. arenosa*. The upstream primer (see Fig. 1*A*) was 5′-TCGGTACCGAGTTTAGGATGTCAAGT-3′; the 18S primer was 5′-GCATATGACTACTGGCAGGATCAACC-3′. PCR products were cloned in pBluescript plasmids (Stratagene). Multiple clones were sequenced.

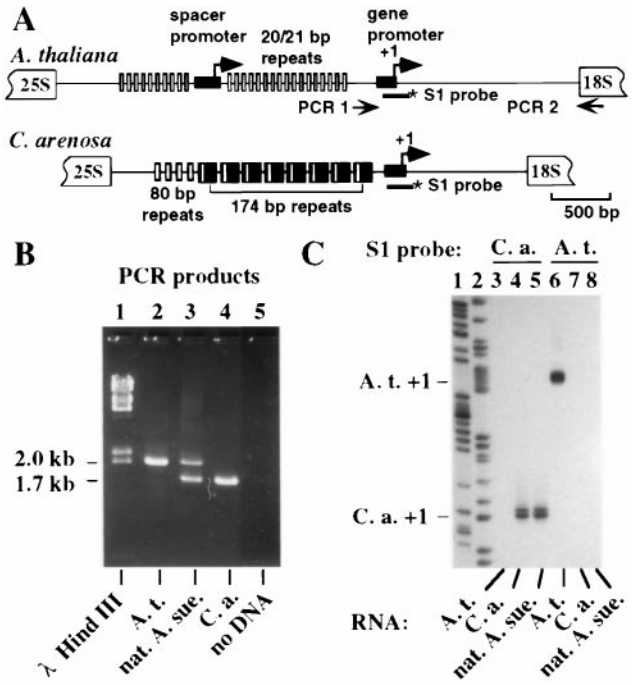

FIG. 1. Nucleolar dominance in *A. suecica*. (*A*) Organization of the intergenic spacers separating 25S and 18S rRNA coding sequences of adjacent rRNA genes. *A. thaliana* intergenic spacers are longer and contain repetitive elements smaller than those located in *C. arenosa* spacers. (*B*) Ethidium bromide-stained agarose gel of PCR products using the primers depicted in *A* and *A. suecica*, *A. thaliana,* and *C. arenosa* genomic DNA. *Hin*dIII-digested λDNA served as size markers in lane 1. The PCR reaction in lane 5 was performed with both primers but no genomic DNA. (*C*) Detection of rRNA transcripts by using the S1 nuclease protection assay. *A. thaliana*, *C. arenosa,* and natural *A. suecica* rRNA transcripts were detected with *C. arenosa*- (lanes 3–5) or *A. thaliana*-specific (lanes 6–8) DNA probes. Dideoxynucleotide sequencing reactions served as size markers in lanes 1 and 2.

Southern Blot Hybridization. Restriction endonuclease-treated genomic DNA was subjected to agarose gel electrophoresis and blotted (23) to ZetaProbe membranes (Bio-Rad). Filters were hybridized (24) to radioactive probes (see figure legends) labeled by random hexamer priming (25).

S1 Protection Assay. rRNA transcripts were detected by S1 nuclease protection as described previously (26). Briefly, 5 μg of total RNA was hybridized to 5′ end-labeled DNA fragments spanning the transcription start site. The *A. thaliana* probe was an *Sph*I-*Eco*RV (−115 to +96) fragment labeled at +96; the *C. arenosa* probe was an *Sph*I-*Bsp*E I fragment (−111 to +59) labeled at +59. RNA/DNA probe hybrids were treated with S1 nuclease (150 units/ml, 37°C, 30 min). Digestion products were resolved on a sequencing gel and exposed to x-ray film. The size of protected products corresponds to the distance from the labeled nucleotide to the transcription start site, +1. S1 probes were used in excess over RNA such that the amount of protected product was proportional to the amount of RNA transcript.

Transient Expression. Protoplasts of sterile-grown 14- to 21-day-old *A. thaliana* and *C. arenosa* plants were transfected as described previously (26, 27). Briefly, 5×10^6 protoplasts were transfected with 50 pmols of supercoiled plasmid containing an *A. thaliana* or a *C. arenosa* rRNA minigene. Following transfection, protoplasts were incubated ≈24 hr. RNA was then purified and analyzed by S1 nuclease protection. *A. thaliana* S1 probes were end-labeled in plasmid sequences such that only minigene transcripts would be detected (27). For pAt1 5′Δ−520 (27), the probe was the *Sph*I–*Bam*HI fragment labeled at the *Bam*HI site; for pAt1 5′Δ-2590/3′Δ+6 (27), the *Sph*I–*Bss*H II fragment was labeled at the *Bss*H II site. The *C. arenosa* probe was the *Sph*I–*Bsp*E I fragment described previously.

RESULTS

***A. thaliana* rRNA Genes Are Silent in *A. suecica*.** *A. suecica* is an allotetraploid derived from *A. thaliana* and *C. arenosa*. *A. thaliana* rRNA genes have been sequenced (28–31) and the intergenic spacer sequence of *C. arenosa* was determined recently (Hayworth and Schaal, personal communication). Based on these data, we designed PCR primers to amplify *A. thaliana* and *C. arenosa* rRNA gene sequences (see Fig. 1*A*). PCR amplification of *A. thaliana* genomic DNA yielded a ≈2.0-kb product (Fig. 1*B*, lane 2) whereas a ≈1.7-kb product was obtained by using *C. arenosa* DNA (Fig. 1*B*, lane 4). PCR amplification of *A. suecica* genomic DNA yielded both 1.7- and 2.0-kb products (lane 3) in similar amounts.

A. suecica PCR products were cloned and sequenced from −265 to ≈+150. *A. thaliana*- and *C. arenosa*-type clones were obtained in approximately equal numbers. Their sequences perfectly matched *A. thaliana* or *C. arenosa* rRNA gene sequences downstream of the transcription start site (+1). This homogeneity allowed the same S1 nuclease protection probes to detect rRNA transcripts in *A. suecica* or the appropriate progenitor species (Fig. 1*C*). By using the *C. arenosa* probe (lanes 3–5), a 59-nucleotide product corresponding to accurately initiated transcripts was detected with *C. arenosa* (lane 4) and *A. suecica* (lane 5) RNA, but not *A. thaliana* RNA (lane 3), verifying the specificity of the probe. Aliquots of the same RNA samples were tested using the *A. thaliana* probe (lanes 6–8). A 96-nucleotide-protected probe fragment was detected with *A. thaliana* RNA (lane 6), but not with *C. arenosa* RNA (lane 7), as expected. Using *A. suecica* RNA, no *A. thaliana* rRNA gene transcripts were detected (lane 8). We conclude that *C. arenosa* rRNA genes are dominant in *A. suecica*.

Stochastic Onset of Nucleolar Dominance in Synthetic *A. suecica*. We have shown that nucleolar dominance occurs in

natural and synthetic strains (32) of *Brassica napus*, *B. juncea* and *B. carinata* allotetraploids (17) but F_3–F_5 generations of synthetic lines were the earliest available for testing (32). Using the *Arabidopsis* system, we asked when dominance is first established. Chromosome counts in meiotic cells of *C. arenosa* 9509 indicated that this and several other races tested are tetraploid (L.C. *et al.*, unpublished work), most likely autotetraploids, because only one type of rRNA gene was detected on sequencing multiple PCR clones (Z.J.C., unpublished work). Autotetraploid *A. thaliana* were generated by colchicine treatment and crossed with *C. arenosa* to recreate synthetic allotetraploid *A. suecica* (Fig. 2). Four self-fertile SAS plants were obtained, designated SAS-1 through SAS-4. *C. arenosa* transcripts were detected in all SAS plants (Fig. 3, lanes 5, 6, 11, 12) at levels similar to the *C. arenosa* control (lane 7). In contrast, expression of *A. thaliana* rRNA genes was variable. In SAS-2 and SAS-4, only trace amounts of *A. thaliana* transcripts could be detected in long exposures (lanes 3 and 4). However, in SAS-1, *A. thaliana* transcripts were as abundant as in the *A. thaliana* control (compare lanes 9 and 8). *A. thaliana* transcripts were expressed at moderate levels (≈40%) in SAS-3 (lane 10). Although the trend is toward dominance of *C. arenosa* genes in SAS plants, as in natural *A. suecica*, at least three epigenetic states for the rRNA genes are revealed in these newly formed allotetraploids: complete dominance, partial dominance, and codominance. PCR and Southern blot analyses of SAS plants confirmed the presence of *A. thaliana* and *C. arenosa* rRNA genes in similar abundance (Z.J.C., data not shown).

To determine whether the variable rRNA gene expression states observed in F_1 plants persist in subsequent generations, we examined F_2 siblings of self-pollinated SAS-1 and SAS-2 plants (Fig. 4). In SAS-1, *A. thaliana* and *C. arenosa* were codominant (see Fig. 3). However, in all F_2 sibs of SAS-1, *C. arenosa* rRNA genes were now dominant, with only trace amounts of *A. thaliana* transcripts detected (Fig. 4*A*, compare

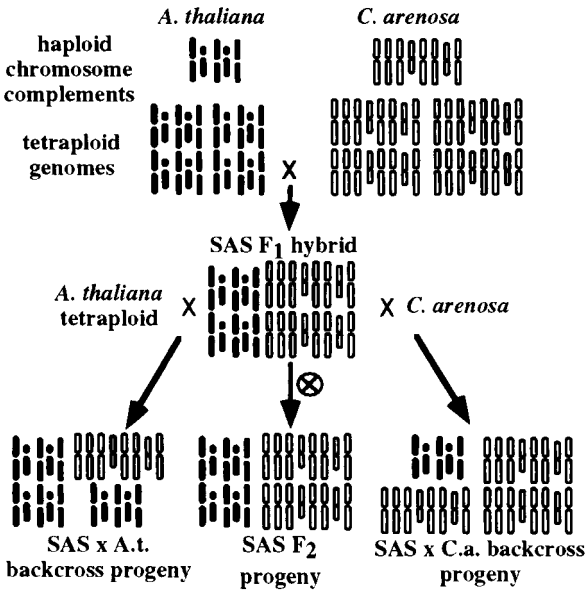

FIG. 2. Creation of synthetic *A. suecica* allotetraploids and backcross progeny. *A. thaliana* is typically diploid, with a haploid complement of five chromosomes. *C. arenosa* is a natural tetraploid with a haploid complement of eight chromosomes. Tetraploid *A. thaliana* was used as the maternal parent in a cross with *C. arenosa*, yielding SAS plants. SAS F_2 progeny were obtained from self-pollinated flowers. Alternatively, flowers were emasculated and manually pollinated with tetraploid *A. thaliana* or *C. arenosa* pollen. Thus progeny with 3:1, 2:2 (F_2), or 1:3 *A. thaliana* to *C. arenosa* genome complements were obtained from the same mother plant.

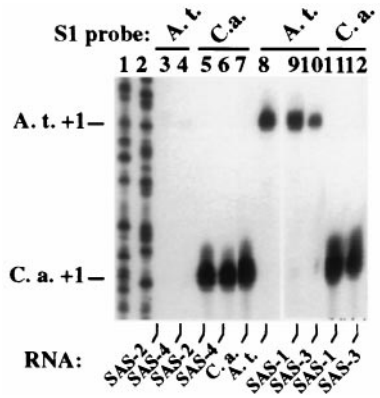

FIG. 3. Variable severity of nucleolar dominance in SAS F_1 hybrids. Total RNA from SAS, *A. thaliana,* and *C. arenosa* plants (lanes 7 and 8) was assayed by using S1 nuclease protection with *A. thaliana*- (lanes 3, 4, 8–10) or *C. arenosa*-specific probes (lanes 5–7, 11, 12). DNA sequencing reactions (lanes 1 and 2) served as size markers.

lanes 1–6 with lanes 9–14). In SAS-2, *C. arenosa* rRNA genes were already dominant in the F_1 generation, with only a trace of *A. thaliana* rRNA transcription detectable (see Fig. 3). In all F_2 sibs of SAS-2, *A. thaliana* transcripts were not detectable even in trace amounts (Fig. 4*B*). *A. thaliana* and *C. arenosa* rRNA gene PCR products were obtained in similar abundance in SAS-1 and all F_2 progeny, indicating that *A. thaliana* rRNA genes were not lost or underrepresented in F_2 plants (data not shown). Collectively, these data show that nucleolar dominance occurs in newly formed *A. suecica* but two generations are necessary for its complete establishment.

The Direction of Nucleolar Dominance Is Subject to Gene or Genome Dosage Effects. To investigate parental gene dosage

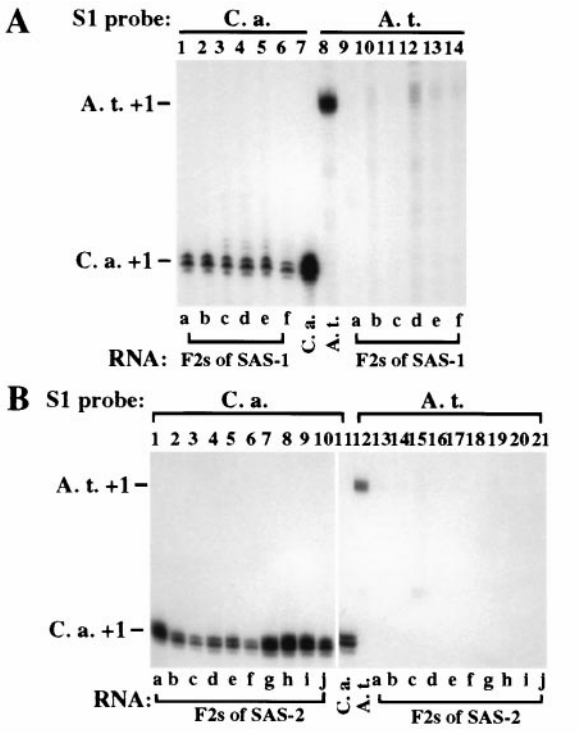

FIG. 4. Two generations are needed for establishment of nucleolar dominance in some SAS lines. (*A*) SAS-1, in which *A. thaliana* and *C. arenosa* rRNA genes were codominant, was self-pollinated to generate F_2 plants. rRNA transcripts in six F_2 siblings and *C. arenosa* and *A. thaliana* controls (lanes 7, 8) were detected using *C. arenosa*- (lanes 1–7) or *A. thaliana*-specific (lanes 8–14) S1 probes. (*B*) F_2 progeny of SAS-2 were analyzed in the same way.

effects on nucleolar dominance, SAS plants were backcrossed to *C. arenosa* or tetraploid *A. thaliana*. In five siblings derived from SAS-2 backcrossed to *A. thaliana*, *A. thaliana* transcripts were detected at levels (Fig. 5*A*, lanes 2–6, top autoradiogram) comparable to the *A. thaliana* control (lane 1, top), whereas in five siblings from a backcross to *C. arenosa*, *A. thaliana* transcripts were not detected (lanes 7–11, top; the weak signal in lane 7 does not correspond to the *A. thaliana* start site and was because of incomplete probe digestion). Examination of *C. arenosa* rRNA gene expression in the SAS-2 × *A. thaliana* backcross progeny yielded interesting results (Fig. 5*A*, bottom left autoradiogram). Only trace amounts of *C. arenosa* transcripts were detected in sibs a and d (lanes 2 and 5). *C. arenosa* transcripts were detected at low levels in sibs c and e (lanes 4 and 6). In sib b, *arenosa* transcripts were abundant (lane 3) but below control levels (lane 1). Overall, the data suggest that in *A. suecica* × *A. thaliana* backcross progeny, the trend is reversed toward dominance of *A. thaliana* genes, although the extent of dominance is variable, reminiscent of the results with the initial synthetic *A. suecica* plants (see Fig. 3). Ethidium-stained RNA gels showed that the variable *C. arenosa* transcript levels in Fig. 5*A* could not be explained by differences in RNA quantity or quality (data not shown). Southern blotting confirmed that both parental sets of rRNA genes were present (Fig. 5*B*). The presence of *A. thaliana* rRNA genes in SAS-2 and its backcross progeny (*Top*, lanes 3–8) was confirmed by using a *A. thaliana*-specific intergenic spacer probe (compare lanes 1 and 2). The equivalent *C. arenosa* probe crossreacted weakly with *A. thaliana* rRNA gene fragments (*Bottom*, compare lanes 1 and 2), but fragment size differences allowed confirmation of *C. arenosa* rRNA genes in SAS-2 and its backcross progeny (lanes 3–8). PCR analyses supported the Southern blot analysis results (not shown). Collectively, these data suggest that the differential expression of *C. arenosa* rRNA genes in SAS-2 × *A. thaliana* backcross progeny cannot be explained by defects in the transmission of *C. arenosa* rRNA genes.

Transient Expression Argues Against Species-Specific Transcription Factors. Coordinate silencing of parental sets of rRNA genes could be accomplished by silencing a species-specific RNA polymerase I transcription factor (9). To test this possibility, we performed reciprocal transfections of cloned *A. thaliana* and *C. arenosa* rRNA minigenes into protoplasts of each species (Fig. 6). The *C. arenosa* minigene was expressed in *A. thaliana* (lane 1) and did not outcompete a *A. thaliana* minigene when both were cotransfected (compare lanes 3 and 4). Likewise, a *A. thaliana* minigene was transcribed both in *C. arenosa* (lane 5) and *A. suecica* (lane 6), despite the fact that the endogenous *A. thaliana* genes are silenced in *A. suecica*.

Cytosine Methylation Enforces rRNA Gene Silencing, as in *Brassica*. In our previous work with *Brassica*, a genus closely related to *Arabidopsis*, inhibiting cytosine methylation (with aza-dC) or histone deacetylation (with trichostatin A or sodium butyrate) derepressed silenced rRNA genes subjected to nucleolar dominance (33). In agreement with these results, *A. thaliana* rRNA transcripts were induced to 40% or 100% of control levels in natural *A. suecica* plants germinated on media containing aza-dC (Fig. 7, compare lanes 4 and 5 to lane 2). *C. arenosa* transcripts were also up-regulated 2- to 3-fold at the highest level of aza-dC (compare lane 8 to lanes 1 and 6). These results do not provide mechanistic insights beyond our previous study but confirm that *A. thaliana* genes in *A. suecica* are not defective, but are silenced.

Verification that aza-dC altered cytosine methylation levels in *A. suecica* was obtained using the restriction endonucleases *Hpa*II or *Msp*I, both of which cut CCGG motifs. *Hpa*II will not cut if the inner C is methylated and cuts very inefficiently if the outer C is methylated. *Msp*I will cut if the inner, but not the outer, C is methylated. Southern blot hybridization using a probe that hybridizes to all *A. suecica* rRNA genes in the conserved ≈2-kb region from the promoter to the 18S rRNA coding sequences showed that without aza-dC treatment, ≈50% of the crosshybridizing signal following *Hpa*II digestion ranged in size from 6- to 23-kb (Fig. 7*B* lane 1). This signal corresponds to *A. thaliana* and *C. arenosa* genes methylated at most or all CCGGs (each gene is ≈10 kb in length). The DNA is more susceptible to *Msp*I cleavage (lane 4), as expected. Aza-dC treatment caused increased susceptibility to *Hpa*II and

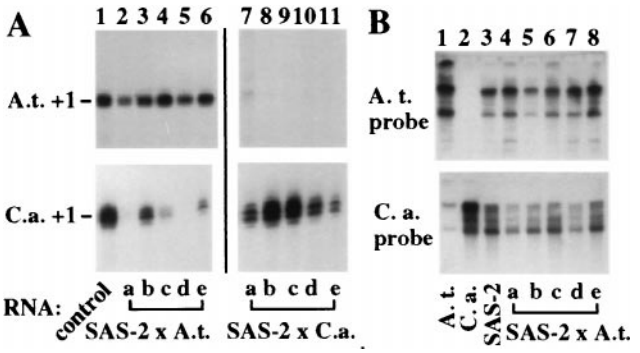

FIG. 5. The direction of nucleolar dominance can be switched. SAS-2, which displayed complete dominance of *C. arenosa* over *A. thaliana* rRNA genes (see Fig. 3) was the maternal parent for backcrosses to tetraploid *A. thaliana* or *C. arenosa*. (*A*) Five backcross sibs from each cross were assayed for rRNA gene expression using *A. thaliana*- (*Top*) or *C. arenosa*-specific (*Bottom*) S1 probes. *A. thaliana* or *C. arenosa* controls are in lane 1 of the relevant autoradiograms. (*B*) Southern blot analysis of *Bss*H II-digested DNA of *A. thaliana* (lane 1), *C. arenosa* (lane 2), SAS-2 (lane 3), or the backcross sibs (lanes 4–8) tested in *A*. The filter was first hybridized to an *A. thaliana*-specific rRNA gene intergenic spacer probe (*Top*), then was stripped and reprobed (49) with a similar *C. arenosa* probe (*Bottom*). The former corresponded to −2,590 to +6 of plasmid pAt1 5′Δ−2,590/3′Δ+6 (27); the latter spanned −2,316 to +33 of pCa1.

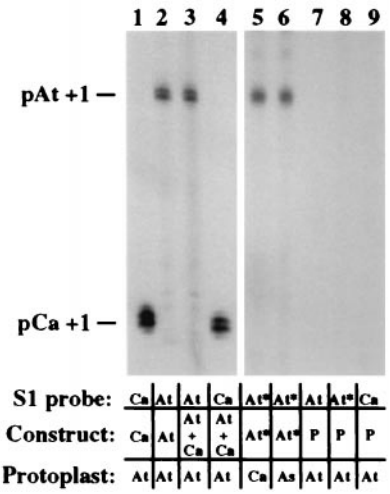

FIG. 6. *A. thaliana* and *C. arenosa* rRNA minigenes are transcribed in the other species. *A. thaliana* (At), *C. arenosa* (Ca) or *A. suecica* (As) protoplasts were transfected with 50 pmols of pBluescript plasmid containing *C. arenosa* rRNA gene promoter sequences (pCa; sequences −265 to ≈+1,700) or one of two *A. thaliana* minigenes, pAt or pAt*. Constructs pAt and pAt* correspond to minigenes pAt1 5′Δ−520 (sequences −520 to +92) and pAt1 5′Δ−2,590/3′Δ+6 (−2,590 to +6), respectively (27). Transcripts were detected with minigene-specific S1 probes. Transcripts in lanes 3 and 4 derive from an experiment in which 50 pmols each of pAt and pCa were cotransfected and half of the isolated RNA was hybridized with the At (lane 3) or Ca (lane 4) probe. Data in lanes 1–4 and 5–9 are derived from the same exposure of a single autoradiogram. Note that no transcripts are detected in protoplasts transfected only with pBluescript (P) DNA (lanes 7–9).

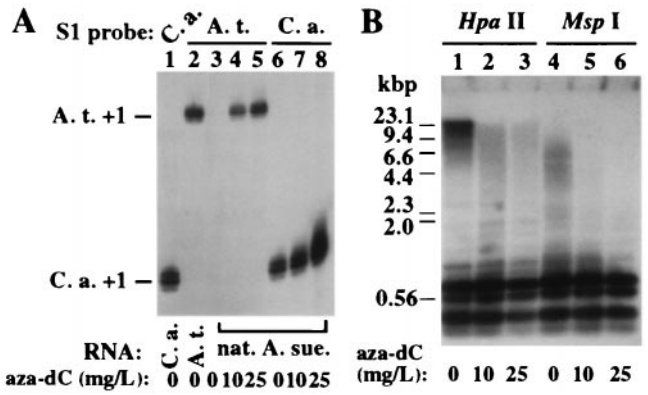

FIG. 7. Derepression of *A. thaliana* rRNA genes in natural *A. suecica* using 5-aza-2′ deoxycytidine (aza-dC). Approximately 30 *A. suecica* seeds were surface-sterilized and germinated on medium containing 0, 10, or 25 mg/L, as described previously (33). (*A*) Total RNA from 3-week-old plantlets was purified and analyzed by S1 nuclease protection with *A. thaliana*- (lanes 2–5) or *C. arenosa*-specific (lanes 1, 6–8) probes. *C. arenosa* and *A. thaliana* control reactions are in lanes 1 and 2. (*B*) Southern blot analysis of DNA from control and treated plants following digestion with *Hpa*II (lanes 1–3) or *Msp*I (lanes 4–6) and hybridization to an *A. thaliana* probe spanning the ≈2-kb region from −365 to the beginning of the 18S rRNA coding sequences. This probe detects both *A. thaliana* and *C. arenosa* rRNA genes.

*Msp*I cleavage, confirming loss of cytosine methylation (lanes 2, 3, 5, 6).

Using Southern blotting and a variety of probes and methylation-sensitive restriction endonucleases, we have been unable to identify specific restriction sites in *Arabidopsis* (or *Brassica*) rRNA gene promoters or intergenic spacers whose methylation state correlates with gene activity or silencing. This observation contrasts with studies of nucleolar dominance in wheat, triticale, and maize (16, 34, 49). The overall degree of *A. thaliana* rRNA gene methylation, estimated by resistance to *Hpa*II digestion, is also not different in *A. thaliana*, natural or synthetic *A. suecica*, or SAS F_2 or backcross progeny, despite their varying degrees of *A. thaliana* rRNA gene expression (data not shown). It is unknown currently whether demethylation of the rRNA genes or a distinct regulatory locus is responsible for the derepression of silenced rRNA genes by aza-dC (see *Discussion* and ref. 33).

DISCUSSION

Despite a long history of cytological and cytogenetic descriptions, few studies have examined nucleolar dominance at a transcriptional level (8, 17, 33). The variable severity of nucleolar dominance we observed in newly formed hybrids contrasts with the longstanding idea that nucleolar dominance is the same in all individuals for a particular cross (2). Furthermore, our study shows that establishment of nucleolar dominance can require multiple generations. This requirement might have gone unnoticed in previous studies using diploid species because their dihaploid F1 progeny are often sterile due to defects in chromosome pairing and segregation. For allotetraploids (amphidiploids) such as *A. suecica*, which contain diploid chromosome complements from both progenitors, infertility is less problematic.

Our most important finding is that gene or genome dosage effects can switch the direction of nucleolar dominance, negating the truism that dominance is independent of rRNA gene dosage or ploidy (9, 10). An effect of genome dosage on the number of nucleoli visible at interphase in allopolyploid *Ribes* (gooseberry and black currant) hybrids was reported, suggesting that dominance could be overcome by increasing the number of underdominant NORs (35). Studies in wheat have shown also that NOR activity is variable in different chromosome addition lines, suggesting that genes unlinked to the NORs can affect their activity (18, 36). However, the demonstration that normally dominant rRNA genes can be made underdominant is unprecedented.

Dominance switching is also at odds with the prevailing hypothesis that dominant rRNA genes outcompete underdominant genes for transcription factors because of higher protein binding affinities (9, 14, 19). Such affinities, described by equilibrium binding constants, should be invariant. Indeed, the presumed insensitivity of nucleolar dominance to gene or genome dosage effects has been interpreted as evidence that rRNA genes are always in excess over transcription factors, such that the genes with highest binding affinities are always dominant. It follows that decreasing the dosage of dominant genes might allow transcription factors to become available to underdominant rRNA genes, but dominant genes should never fall silent. Our demonstration of dominance switching argues that rRNA genes are not independently regulated based on their intrinsic affinities for transcription factors. Consistent with this conclusion from genetic experiments, dominant and underdominant *Brassica* rRNA genes compete equally for transcription factors (Frieman and C.S.P., unpublished data) in the *in vitro* transcription system we have developed (37).

Coordinate control of parental sets of rRNA genes might be accomplished by controlling the expression of a species-specific transcription factor. However, our transient expression results argue against this possibility. The transient expression results suggest further that nucleolar dominance is a chromosomal phenomenon that is enforced, and possibly established, independent of transcription factor availability. Supporting evidence is that in *Drosophila* hybrids, chromosomal rearrangements adjacent to the NORs of *D. melanogaster* eliminate suppression of *D. simulans* nucleolus formation in trans without reducing *D. melanogaster* rRNA gene or nucleolus expression (38). Therefore, expression of rRNA genes at dominant NORs is not sufficient to cause repression of an underdominant NOR, as predicted by the limiting transcription factor hypothesis (38). Durica and Krider's observations also show that loci flanking the NORs play a role in their regulation (38). Being closely linked to the NORs, it is possible that these flanking loci are the true determinants of nucleolar dominance rather than structural features of the rRNA genes within the dominant NORs.

Variable rRNA gene silencing in F_1 hybrids suggests a stochastic component to the establishment of nucleolar dominance. Furthermore, completion of nucleolar dominance by the F_2 generation implies that silencing is cumulative and is not erased at meiosis. *De novo* cytosine methylation is a candidate DNA modification that might explain the stochastic onset and progressive establishment of nucleolar dominance (39–43) and is consistent with the derepression of silenced rRNA genes by aza-dC. However, we have been unable to correlate rRNA gene activity with overall rRNA gene methylation levels or methylation of specific restriction sites in either *Brassica* (33) or *Arabidopsis* (this study). Perhaps critical methylated cytosines have gone unnoticed using this approach. However, we have also found that rRNA gene transcription *in vitro* is insensitive to cytosine methylation (Frieman and C.S.P., unpublished data). Furthermore, histone deacetylase inhibitors derepress silenced rRNA genes without detectably altering rRNA gene methylation *in vivo* (Z.J.C. and C.S.P., unpublished work). These results argue against models in which hypermethylation of underdominant rRNA gene promoters directly blocks transcription factor binding (44). However, it is possible that methylation acts indirectly via recruitment of methylcytosine-binding protein complexes that include histone deacetylase activity (45, 46). Histone deacetylation, in turn, might render the chromatin structure of rRNA gene promoters

inaccessible to transcription factors. The latter model is consistent with the derepression of silenced rRNA genes caused by histone deacetylase inhibitors (33), the lack of synergy between these inhibitors and aza-dC (33), and the fact that histone deacetylase inhibitors have no detectable effect on rRNA gene methylation levels (Z.J.C. and C.S.P., unpublished work).

Another possibility is that aza-dC and histone deacetylase inhibitors derepress silenced rRNA genes by activating a regulatory locus that controls the transcriptional competence of the NOR. Involvement of other loci is consistent with studies showing loss of nucleolar dominance because of chromosome substitution in wheat and triticale (18, 36) or because of chromosome rearrangements in *Drosophila* (38) and barley (47). Our current study suggests that such hypothetical regulatory loci are unlikely to encode species-specific transcription factors. Beyond this conclusion, our current understanding falls short of considering other candidate loci. The availability of *A. thaliana* hypomethylation mutants (48) and other *Arabidopsis* genetic resources should make *A. suecica* a promising model system for further study.

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