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Transcriptional analysis of nucleolar dominance in polyploid plants: Biased expression/silencing of progenitor rRNA genes is developmentally regulated in *Brassica*

(rDNA/nucleolus/RNA polymerase I/gene silencing/epigenetic phenomena)

Z. JEFFREY CHEN AND CRAIG S. PIKAARD*

Biology Department, Washington University, Campus Box 1137, One Brookings Drive, St. Louis, MO 63130

Communicated by Peter H. Raven, Missouri Botanical Garden, St. Louis, MO, December 31, 1996 (received for review December 2, 1996)

ABSTRACT **Nucleolar dominance is an epigenetic phenomenon that describes the formation of nucleoli around rRNA genes inherited from only one parent in the progeny of an interspecific hybrid. Despite numerous cytogenetic studies, little is known about nucleolar dominance at the level of rRNA gene expression in plants. We used S1 nuclease protection and primer extension assays to define nucleolar dominance at a molecular level in the plant genus *Brassica*. rRNA transcription start sites were mapped in three diploids and in three allotetraploids (amphidiploids) and one allohexaploid species derived from these diploid progenitors. rRNA transcripts of only one progenitor were detected in vegetative tissues of each polyploid. Dominance was independent of maternal effect, ploidy, or rRNA gene dosage. Natural and newly synthesized amphidiploids yielded the same results, arguing against substantial evolutionary effects. The hypothesis that nucleolar dominance in plants is correlated with physical characteristics of rRNA gene intergenic spacers is not supported in *Brassica*. Furthermore, in *Brassica napus*, rRNA genes silenced in vegetative tissues were found to be expressed in all floral organs, including sepals and petals, arguing against the hypothesis that passage through meiosis is needed to reactivate suppressed genes. Instead, the transition of inflorescence to floral meristem appears to be a developmental stage when silenced genes can be derepressed.**

Ribosomal RNA genes (rRNA genes, rDNA) in eukaryotes are tandemly arrayed in hundreds (to thousands) of copies at chromosomal loci known as nucleolus organizer regions (NORs) (1). Each rRNA gene can be transcribed within the nucleolus by RNA polymerase I to produce a primary transcript that is processed to form the 18S, 5.8S, and 25–27S rRNAs (the size depends on species). To form ribosomes, these RNAs are assembled with 5S rRNA transcribed by RNA polymerase III and ≈80 proteins whose mRNAs are transcribed by RNA polymerase II. Because ribosome production directly affects the protein synthetic capacity of the cell, proper regulation of rRNA gene transcription is critical (2–9).

The study of rRNA gene regulation in eukaryotes has focused primarily on *in vitro* analyses of transcriptional activation of individual rRNA genes and the biochemical characterization of transcription factors (6). However, cytogenetic evidence for rRNA gene regulation on a larger scale has accumulated for nearly 70 years on the basis of studies of nucleolar dominance. Nucleolar dominance occurs in organisms as diverse as insects, amphibians, and mammals (10) but was first described in the plant genus *Crepis* (11–13), followed by studies in *Salix*, *Ribes*, *Solanum*, *Hordeum*, *Avena*, *Agropyron*, *Triticum*, and *Zea*, and in intergeneric crosses such as *Triticale* (wheat × rye; reviewed in refs. 10 and 14). In wheat, changes in the methylation status and DNase I hypersensitivity of dominant and underdominant (the term *recessive* is inappropriate) rRNA genes suggest that inactive genes are packaged into a transcriptionally inactive chromatin structure (15, 16). However, no direct analysis of rRNA gene promoter activity in plants exhibiting nucleolar dominance has yet been reported.

In this study, we developed molecular probes to examine nucleolar dominance in well-characterized *Brassica* allopolyploids. We show that rRNA gene promoters inherited from only one progenitor are active in vegetative tissues and that dominance is independent of maternal effect and ploidy. Surprisingly, rRNA genes silent in vegetative tissues of *Brassica napus* are expressed in flowers, suggesting that the genetic mechanisms responsible for nucleolar dominance are developmentally regulated in each generation after polyploidization.

MATERIALS AND METHODS

Brassica seeds were obtained from the Crucifer Genetics Cooperative (CrGC), Madison, WI. Stock numbers were as follows: *Brassica rapa* (genome: aa, CrGC no. 1–1), *Brassica nigra* (bb, CrGC no. 2–1), *Brassica oleracea* (cc, CrGC no. 3–1), *Brassica juncea* (aabb, CrGC no. 4–1), *B. napus* (aacc, CrGC no. 5–1), and *Brassica carinata* (bbcc, CrGC no. 6–1). The synthetic tetraploids *B. carinata* (bbcc, *B. nigra* × *B. oleracea*, F_3, and *B. oleracea* × *B. nigra*, F_4), *B. juncea* (aabb, *B. rapa* × *B. nigra*, F_5, and *B. nigra* × *B. rapa*, F_5), and *B. napus* (aacc, *B. rapa* × *B. oleracea*, F_5, and *B. oleracea* × *B. rapa*, F_5) were provided by Thomas Osborn (17). *B. napus*-like hexaploid lines (aacccc, R1 and R2) were provided by Xingyong Wu (Oil Crop Institute, Chinese Academy of Agricultural Sciences). Plants were grown in Terra-Lite soil mix (Grace Horticultural Products) in the greenhouse or in growth chambers.

Genomic DNA was purified (18) using pooled tissue from 5–10 plants of the same genotype. The polymerase chain reaction (PCR) was used to amplify *Brassica* promoter regions using the primers 5′-TCGGTACCGAGTTTAGGATGTCAAGT-3′ (matching *B. oleracea* sequences from −265 to −248; see ref. 19) and 5′-TAGGATCCGGAAAAGTCGCCGGAAAAG-3′ (+142 to +163; +1 is the transcription start site). Sequences matching the primers are highly conserved in *Brassica.* PCR involved denaturation at 95°C, 2 min; 25–30 cycles of 94°C (45 sec), 60°C (30 sec), 72°C (120 sec); and a final incubation at 72°C for 6 min. Amplified DNA was cloned in pBluescript plasmids (Stratagene) and multiple independent clones were sequenced on both strands.

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Abbreviation: NOR, nucleolus organizer region.

*To whom reprint requests should be addressed.

RNA was extracted by using 4.5 M guanidinium thiocyanate followed by precipitation with lithium chloride (20) or was extracted in a buffer containing NaCl and SDS (21). For the analyses of Figs. 2–5, leaf tissue from three to five plants (3–4 weeks old) was pooled for RNA isolation. No variation among individual plants was detected (not shown). Organ-specific RNAs (see Fig. 6) were purified from tissue harvested on the same day from the same plant.

S1 nuclease protection was performed as described previously (22, 23) using a 5′-end-labeled restriction fragment spanning the transcription start site hybridized with 15 μg of total RNA. DNA·RNA hybrids were treated with S1 nuclease (150 units/ml, 30 min, 37°C) to digest single-stranded nucleic acid (unhybridized DNA and RNA) and products were resolved on a sequencing gel. The size of a protected probe fragment corresponds to the distance from the transcription start site to the labeled nucleotide. Probes were used in excess over RNA so that the protected product was proportional to the amount of RNA transcript. To detect *B. rapa* transcripts the probe was the *Sph*I (−110) to *Ava*II (+76) fragment; *B. oleracea* and *B. nigra* probes were *Acc*I (−39) to *Ava* II (+103 and +95 in *B. oleracea* and *B. nigra*, respectively) fragments.

Primer extension of rRNA transcripts was carried out as described previously (23), using reverse transcriptase and an excess of the primer 5′-GATTTTAGGGGCAAAATGGTC-CACCGGAA-3′ (see Fig. 1*C*). This 5′-end-labeled primer was also used for dideoxynucleotide DNA sequencing, allowing mapping of transcription start sites by comparison to the sequence ladder.

RESULTS

Cloning rRNA Gene Promoters from Species of the "Brassica Triangle." The *Brassica* genus includes a diverse group of diploid and polyploid species (24). Six are major crops, comprising the species triangle (25) shown in Fig. 1*A*. Diploids include *B. rapa* (also known as *Brassica campestris*; turnip rape, Chinese cabbage, Sarson; genome designated as aa, chromosome number $n = 10$), *B. nigra* (black mustard; bb, $n = 8$), and *B. oleracea* (cabbages, broccoli, cauliflower, Brussels sprouts, kohlrabi, kale, and collards; cc, $n = 9$). The tetraploids derived from these species are *B. juncea* (*B. nigra* × *B. rapa,* brown mustard; aabb, $n = 18$), *B. carinata* (*B. nigra* × *B. oleracea*; Abyssinian mustard; bbcc, $n = 17$), and *B. napus* (*B. oleracea* × *B. rapa*, oilseed rape, canola; aacc, $n = 19$). Haploid rRNA gene numbers in *Brassica* diploids have been estimated to be ≈1,500–3,400 (19). Diploids appear to have two major NORs (26, 27), though minor rRNA gene loci can be detected by fluorescence *in situ* hybridization (28). Physical sizes of *Brassica* NORs are unknown. However, the related species *Arabidopsis thaliana* has approximately 750 rRNA genes per haploid genome located at two NORs, each ≈3.5–4.0 Mbp (29–31). Given the 2- to 4-fold increase in rRNA gene copy number in *Brassica* species relative to *Arabidopsis*, we expect *Brassica* NORs to be 7–14 Mbp.

Promoters and other regulatory elements are located within the intergenic spacers that separate adjacent rRNA genes. On the basis of intergenic spacer sequences of *B. oleracea*, *B. rapa*, and *B. nigra* (19, 32–34), we designed PCR primers to amplify and clone the promoter regions (see Fig. 1*B*). Multiple promoter clones of diploid (Fig. 1*C*) and amphidiploid species were sequenced. Amphidiploids contained the promoters of both progenitor species, as expected (35). For instance, in *B. carinata*, whose tetraploid genome is derived from *B. oleracea* and *B. nigra*, six clones had 100% identity to the promoter of *B. oleracea* and four clones had 100% identity to the *B. nigra* promoter. No sequence polymorphisms were observed in the promoter region sequenced. These data suggest that rRNA gene promoter sequences are unchanged since formation of the polyploid, presumably thousands of years ago (24).

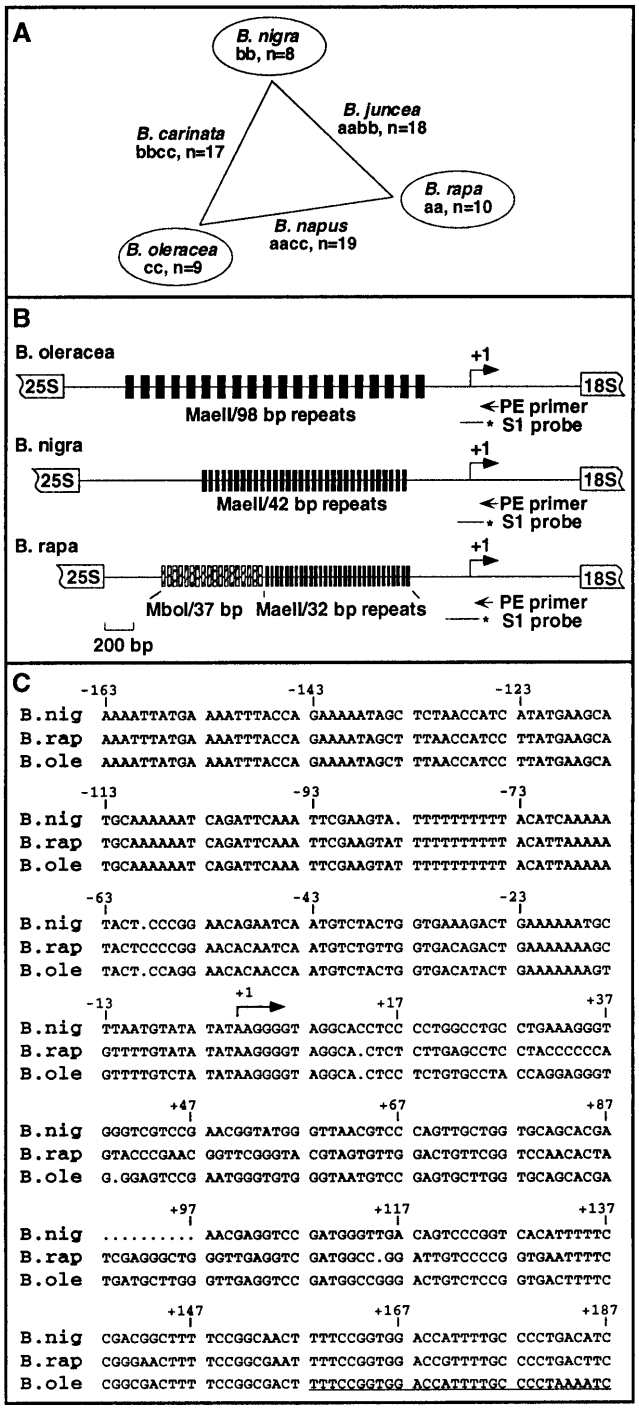

FIG. 1. (*A*) *Brassica* diploids and tetraploids used in this study. The triangle illustrates the genomic relationships among the six species. *B. nigra* is placed at the top and *B. rapa* is placed higher than *B. oleracea* to summarize our finding that the hierarchy of rRNA gene transcriptional dominance is *B. nigra* > *B. rapa* > *B. oleracea*. (*B*) Diagrams of the rRNA gene intergenic spacers of the three diploids. Upstream of the gene promoter (arrows at +1) are repetitive elements that differ in number and size between species. These repeats are putative enhancers based on studies of analogous elements in *Xenopus*, mouse, and *Arabidopsis*. The positions of primer extension (PE) and S1 nuclease protection probes are indicated below the diagrams. (*C*) Comparison of rRNA gene promoter sequences of the three diploid species. The sequences are very similar upstream of the transcription start site and downstream of +100. Underlined sequences are complementary to the primer used for primer extension analyses in Figs. 2 and 3.

Nucleolar Dominance in *Brassica* Tetraploids. *B. oleracea* and *B. nigra* sequences downstream of the transcription initi-

ation site (+1) are different enough that their transcripts can be discriminated by primer extension and S1 nuclease protection assays. Relative to *B. nigra*, *B. oleracea* has 10 bp inserted between +88 and +97 but is missing nucleotides at +13 and +115 in the numbering system of Fig. 1*C*. Using a single primer complementary to rRNAs of both species (underlined in Fig. 1*C*), primer extension with RNA of *B. oleracea* and *B. nigra* yielded products of 185 nt (Fig. 2*A*, lane 7) and 177 nt (lane 5), respectively, as expected. Transcripts initiate at the sequence TATATAAGGG (+1 is in boldface), a sequence very similar to the rRNA transcription start site of *Arabidopsis thaliana* and other plant species (23). However, primer extension of RNA isolated from the tetraploid *B. carinata* yielded only a single product (lane 6) matching that of *B. nigra* rRNA. *B. oleracea* transcripts could not be detected in *B. carinata*, even upon long exposure to x-ray film or a PhosphorImager screen. We estimate that transcripts must be under-represented by more than 200-fold to escape detection.

The results of Fig. 2*A* were obtained with a natural strain of *B. carinata*. To determine if evolutionary changes since polyploid formation play a role in the phenomenon, we tested F_3 and F_4 generations of synthetic *B. carinata* lines created by colchicine-induced chromosome doubling of *B. nigra* × *B. oleracea* F_1 hybrids (17) (Fig. 2*B*). The two lines tested were derived from reciprocal crosses to control for maternal effects. As in natural *B. carinata*, only *B. nigra* rRNA gene transcripts were detected in synthetic lines (lanes 6 and 7). Primer extension results were confirmed using the S1 nuclease protection assay. *B. nigra* rRNA transcripts were readily detected in both natural (Fig. 2*C*, lane 4) and synthetic (lanes 5 and 6) lines of *B. carinata*, but *B. oleracea* transcripts were not detectable (lanes 9–11).

We next characterized nucleolar dominance in the tetraploids *B. juncea* and *B. napus. B. nigra* and *B. rapa* are the progenitor species of *B. juncea*. When the same primer as in Fig. 2 was used, 177-nt and 185-nt primer extension products were detected with *B. nigra* and *B. rapa* RNA (Fig. 3, lanes 5 and 9), respectively, as expected. In both natural *B. juncea* and in the F_5 generation of synthetic *B. juncea* derived from reciprocal crosses, only *nigra*-like rRNA transcripts were detected (Fig. 3, lanes 6–8). *B. rapa* rRNA transcripts were not detectable. These results were confirmed using S1 nuclease protection (data not shown).

The data of Figs. 2 and 3 show that *B. nigra* rRNA genes are dominant over those of *B. oleracea* and *B. rapa*. Consequently, we were interested in determining if dominance or codominance would be observed among *B. rapa* and *B. oleracea* rRNA genes in *B. napus*. In both natural and synthetic *B. napus* (F_5 generations of reciprocal crosses), *B. rapa* transcripts were detected (Fig. 4, lanes 4–6) by S1 nuclease protection. *B. oleracea* transcripts were not detectable in natural *B. napus* (lane 9) but were detectable at low levels (about 2% of the *B. oleracea* control, lane 7, based on phosphorimaging) in synthetic lines (lanes 10 and 11; a 3-fold longer exposure time was needed for lanes 10 and 11 relative to other lanes). These results suggest that, unlike the situation in *B. carinata*, *B. juncea*, and natural *B. napus*, nucleolar dominance is incomplete in leaves of synthetic *B. napus*. Nonetheless, the dominance of *B. rapa* over *B. oleracea* rRNA genes is clear. Genotype differences among the progenitors of natural and synthetic *B. napus* may affect the extent of *B. oleracea* rRNA gene expression.

Nucleolar Dominance Is Unaffected by Ploidy or rRNA Gene Dosage. Restriction fragment length polymorphism (RFLP) analyses suggest that rRNA genes of both parents are present in similar numbers in *Brassica* tetraploids (refs. 35 and 36; R. Peck, Z.J.C., and C.S.P., unpublished results). Therefore, rRNA gene number alone is an unlikely cause of nucleolar dominance. Nonetheless, the incomplete silencing of underdominant genes in synthetic *B. napus* prompted us to test

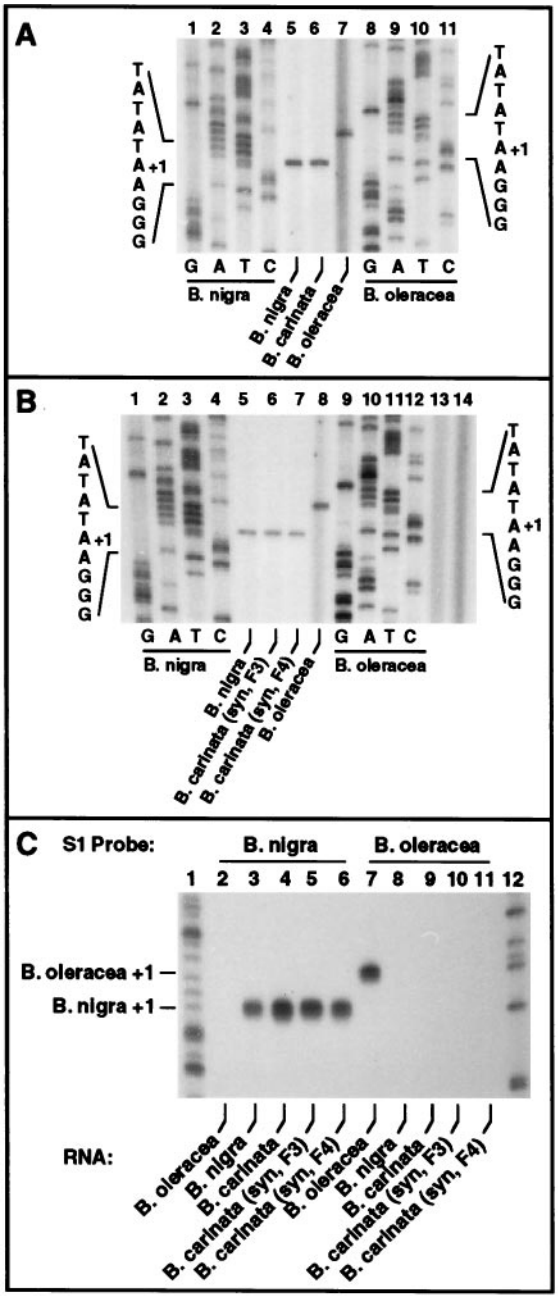

FIG. 2. *B. nigra* rRNA genes are dominant over *B. oleracea* rRNA genes in the tetraploid, *B. carinata*. RNA isolated from *B. nigra* and *B. oleracea* yielded primer extension products of expected sizes (*A*, lanes 5 and 7; *B*, lanes 5 and 8). Primer extension of RNA isolated from natural (*A*, lane 6) or synthetic (*B*, lanes 6 and 7) *B. carinata* lines revealed the presence of *B. nigra*, but not *B. oleracea*, transcripts. Synthetic *B. carinata* lines tested in *B* and *C* are the products of reciprocal crosses, revealing that there is no maternal effect. The same primer was used to generate sequencing ladders from promoter DNA clones (*A*, lanes 1–4, 8–11) allowing the transcription initiation sites (+1) to be accurately mapped. Lanes 13 and 14 in *B* are controls with no RNA and yeast tRNA, respectively, in the primer extension reactions. Primer extension results were confirmed by S1 nuclease protection (*C*) using probes specific for *B. nigra* (*C*, lanes 2–6) or *B. oleracea* (*C*, lanes 7–11) transcripts. Sequencing reaction products were loaded as size markers in lanes 1 and 12.

whether increasing the dosage of *B. oleracea* genes might counteract nucleolar dominance. Two hexaploid lines (F_4 generations) with four haploid genome equivalents from *B. oleracea* and two from *B. rapa* were tested (labeled 6x, R1, and R2 in Fig. 5). *B. rapa* rRNA genes remained dominant in the

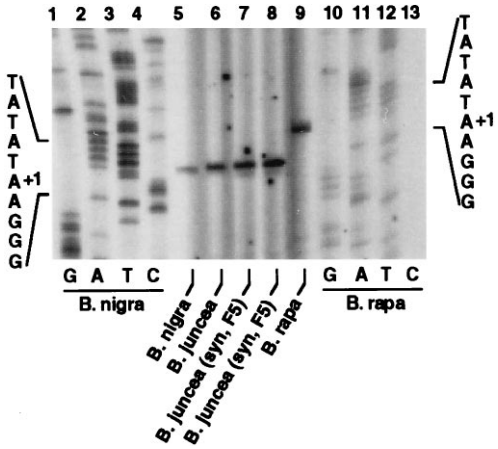

FIG. 3. *B. nigra* rRNA genes are dominant over *B. rapa* rRNA genes in both natural and synthetic lines of the tetraploid *B. juncea*. RNA purified from *B. nigra* (lane 5), *B. rapa* (lane 9), or *B. juncea* (lanes 6–8) was subjected to primer extension using the same primer. Only *B. nigra* transcripts were detected in the tetraploids. RNA from natural *B. juncea* was tested in lane 6; synthetic *B. juncea* lines derived from reciprocal crosses were tested in lanes 7 and 8.

synthetic hexaploid lines (Fig. 5, lanes 1 and 2) with *B. oleracea* transcripts detectable only at low levels (lanes 3 and 4) similar to those observed in the synthetic tetraploids (see Fig. 4). Therefore, nucleolar dominance in *Brassica* appears to be independent of ploidy, in agreement with Navashin's original observations in *Crepis* (11).

Developmentally Programmed Reactivation of Suppressed rRNA Genes. Cytogenetic studies have shown that nucleoli suppressed in diploid root tip cells are active in haploid microspores (1, 13). This has been interpreted to mean that nucleolar dominance is relieved when dominant and underdominant rRNA genes are separated by a nuclear membrane after meiosis (10, 13). With this in mind, we explored the developmental regulation of nucleolar dominance in vegetative and reproductive organs of *B. napus*. Shoot apical meristems of plants such as *Brassica* and *Arabidopsis* undergo several

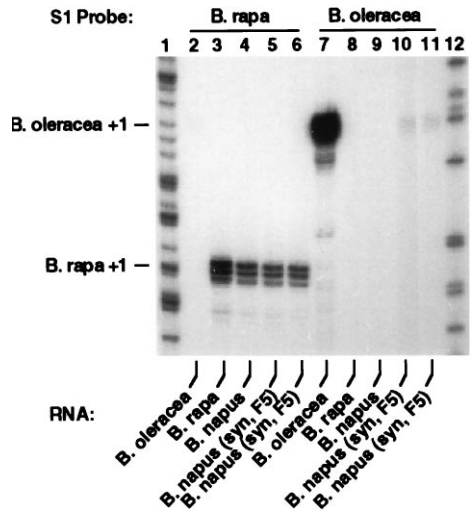

FIG. 4. *B. rapa* rRNA genes are dominant over *B. oleracea* in both natural and synthetic lines of tetraploid *B. napus*. RNA isolated from *B. oleracea*, *B. rapa*, or the tetraploids was subjected to S1 nuclease protection assay using a *B. rapa* probe (lanes 2–6) or a *B. oleracea* probe (lanes 7–11). *B. rapa* transcripts were readily detected in the tetraploids (lanes 4–6). No *B. oleracea* transcripts were detected in natural *B. napus* (lane 8), but trace amounts were detected in the synthetic lines (lanes 10 and 11). DNA sequencing reactions were loaded as size markers in lanes 1 and 12.

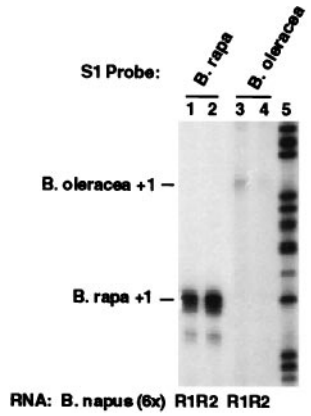

FIG. 5. Nucleolar dominance is independent of rRNA gene dosage and ploidy. Two hexaploid *B. napus*-like lines (R1 and R2) having two diploid sets of *B. oleracea* chromosomes but only one set of *B. rapa* chromosomes were tested to see if doubling the dosage of *B. oleracea* rRNA genes could alter the dominance relationship. S1 nuclease protection assays show that *B. rapa* rRNA genes remain dominant (lanes 1 and 2) as in tetraploid *B. napus* (Fig. 4). Trace amounts of *B. oleracea* transcripts were detectable in the hexaploid lines (lanes 3 and 4). Size markers are shown in lane 5.

developmental transitions (37). As a vegetative meristem, leaves are produced. In response to developmental and environmental cues, a reprogramming of the meristem occurs. The resulting inflorescence meristem produces the elongated flower stalk stem (or "bolt") and specialized cauline leaves attached to the bolt. The inflorescence meristem, in turn, undergoes a final transition to become a floral meristem which produces sepals, petals, stamens, and carpels. Microspores are formed after meiosis in anthers of stamens, and megaspores develop within carpels. Following fertilization, the ovary bearing the embryos elongates to form a silique (seed pod).

From single natural *B. napus* plants (approximately 10 weeks old), vegetative leaves, cauline leaves, flower buds, sepals, petals, anthers, and developing siliques were isolated on the same day. Purified RNA was then tested for the presence of *B. rapa* and *B. oleracea* rRNA transcripts. *B. rapa* transcripts were detected at similar levels in all organs, as expected (Fig. 6, lanes 4–10; all RNA tested is from the same plant). Expression of *B. oleracea* transcripts was not detectable in vegetative leaves (lane 12), in agreement with the results of Fig. 4 (using 3- to 4-week-old plants). Likewise, *B. oleracea* transcripts were not detected in cauline leaves derived from the inflorescence meristem (lane 13). However, in all organs derived from the floral meristem, including immature floral buds, sepals, petals, anthers, and siliques, *B. oleracea* rRNA transcripts were detected at approximately 30–50% of *B. rapa* rRNA gene transcript levels (lanes 14–17). These data suggest that nucleolar dominance is a developmentally regulated phenomenon and that the floral meristem transition may be a stage when rRNA genes can be reactivated. Meiosis and gamete formation are clearly not a prerequisite for reactivation of suppressed genes in *B. napus*.

DISCUSSION

Isozyme studies suggest that most gene loci inherited from two or more progenitor species are codominant in polyploids (38–41); there are relatively few examples of redundant gene silencing (42, 43). Therefore, nucleolar dominance does not appear to be part of a broader genome silencing phenomenon (10). The *Brassica* genus offers a promising system to explore the mechanisms responsible for nucleolar dominance due to the availability of polyploids formed in various combinations from the same diploid progenitors (Fig. 1*A*). With molecular

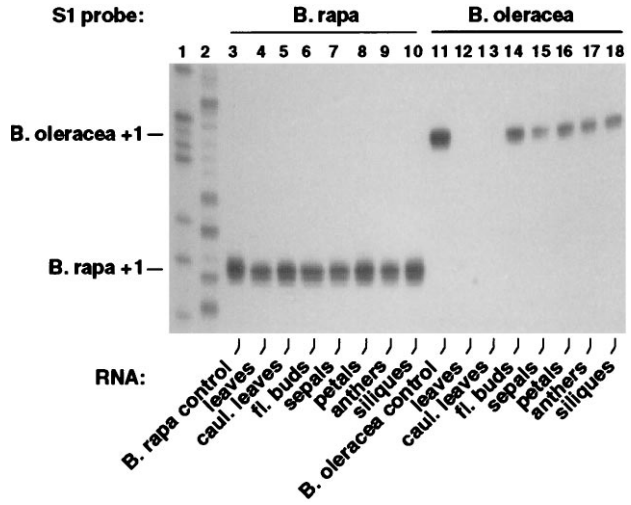

FIG. 6. Underdominant rRNA genes suppressed in vegetative tissues are expressed in floral tissues of *B. napus*. Vegetative leaves, cauline leaves, floral buds, sepals, petals, anthers, and siliques from a single mature *B. napus* plant were harvested on the same day. An equal amount of total RNA from each type of organ was subjected to S1 nuclease protection analysis using probes specific for *B. rapa* (lanes 3–10) or *B. oleracea* (lanes 11–18) rRNA transcripts, as in Fig. 4. RNA from diploid *B. rapa* or *B. oleracea* served as controls (lanes 3 and 11) and show that the two probes were similar in specific activity. Note that *B. oleracea* genes are readily detected in all organs derived from the floral meristem (lanes 14–18) but are not detected in vegetative leaves or cauline leaves (lanes 12 and 13). DNA sequencing reaction products served as size markers in lanes 1 and 2.

probes, our data reveal the dominance hierarchy *B. nigra* > *B. rapa* > *B. oleracea*. Nucleolar dominance in *Brassica* is independent of maternal effect and genome dosage, consistent with Navashin's early cytological observations in *Crepis* (11). Our data are also consistent with available cytological evidence for *Brassica*. Olin-Fatih and Heneen observed only a diploid number of nucleoli in root-tip cells of tetraploid *B. napus* (26), suggesting that nucleolus formation by one set of rRNA genes was repressed. Our results suggest that the *B. rapa* rRNA genes are active, whereas *B. oleracea* genes are repressed, illustrating the utility of molecular analyses to confirm and extend cytological investigations.

An appealing hypothesis to explain nucleolar dominance is that dominant rRNA genes have the most transcriptional enhancers (8, 10). In plants, the evidence supporting this hypothesis is indirect. Hexaploid wheat has multiple NORs (44, 45). The largest (presumably the most active transcriptionally) NOR includes rRNA genes with the longest intergenic spacers (8, 46–48). *Aegilops umbellulata*, a wild relative, has rRNA genes with even longer spacers, and a single NOR-bearing *Aegilops* chromosome in a wheat background can suppress all wheat NORs (46). Most spacer length variation in plants and animals is due to differences in the number of tandemly repeated intergenic spacer elements (2, 8, 9). In *Xenopus*, mouse, and *Arabidopsis* there is biochemical evidence that these repetitive elements function as enhancers of rRNA transcription (22, 49–52). Therefore, a logical deduction is that nucleolar dominance in wheat results from an inequity in enhancer dosage and titration of a limiting transcription factor, analogous to a model proposed for *Xenopus* based on oocyte injection studies (10). However, intergenic spacer characteristics are not correlated with nucleolar dominance in *Brassica. B. oleracea* rRNA genes have the longest intergenic spacers, yet these genes are inactivated when they are in the same nucleus as *B. rapa* or *B. nigra* rRNA genes (see Fig. 1*B*). Conversely, *B. nigra* rRNA genes have the shortest intergenic spacers, yet are dominant over the rRNA genes of the other two species. Likewise, the size of the repetitive region is not correlated with nucleolar dominance, nor is the number of repetitive elements (see Fig. 1). Clearly, simple visual comparisons of spacer characteristics cannot address enhancer activity, highlighting the need for rigorous biochemical tests of enhancer activity and the enhancer imbalance hypothesis.

Other evidence suggests that underdominant rRNA genes are subjected to repression, suggesting that enhancer dosage and transcription factor availability alone are unlikely to explain all aspects of nucleolar dominance. Cytosine methylation is often involved in epigenetic silencing in animals and plants (53–55), and an inhibitor of cytosine methyltransferase, 5-aza-2′-deoxycytidine (aza-dC), can often cause reactivation of suppressed loci (56). Cytological studies have shown that aza-dC can activate suppressed nucleoli subjected to nucleolar dominance in plants (57, 58). Likewise, suppressed *Brassica* rRNA gene promoters are activated by aza-dC (unpublished results). These observations suggest repression of underdominant rRNA genes at the level of chromatin structure, consistent with other studies (15, 16, 59, 60). Experiments are needed to determine if changes in DNA methylation are a cause or effect of nucleolar dominance and to explain how dominant and underdominant genes are first discriminated within the nucleus.

Our study shows that underdominant rRNA genes repressed in vegetative tissues of *B. napus* are expressed in all floral organs, including sepals and petals not involved in gametogenesis. Therefore, the hypothesis that reactivation of silenced genes occurs when dominant and underdominant NORs are segregated by meiosis should be carefully reconsidered (10, 13). RNA can be isolated from virtually any organ or tissue for detection of rRNA transcripts, whereas cytological observations are often limited to favorable cell types such as root tips and pollen mother cells. Reactivation of suppressed nucleoli prior to pollen development may have been missed in prior cytological studies because of this limitation. Alternatively, reactivation might occur at different developmental stages in different species. In any event, we suggest that reactivation of suppressed rRNA genes in *B. napus* is associated with the developmental transition from the inflorescence to floral meristem. Likewise, Honjo and Reeder showed that nucleolar dominance was complete in early embryos of *Xenopus* hybrids, but transcripts from underdominant genes could be detected late in embryonic development and in organs of adult frogs (43). Therefore, in both animals and plants there is evidence that nucleolar dominance is developmentally regulated independent of gamete formation.

It has been argued that plants have more rRNA genes than they need (8). For instance, maize can have 2,500–24,000 rRNA genes per diploid genome (61, 62), most of which are condensed into (presumably) transcriptionally inactive heterochromatin (61, 63). Gene redundancy caused by polyploidization would only exacerbate the excessive number of rRNA genes. This might occur frequently in nature, given that ≈30% of all flowering plants and ≈70% of all grasses are thought to be ancient polyploids (64). An important goal for future studies will be to determine if nucleolar dominance is a hybrid-specific dosage compensation mechanism or a consequence of the same mechanisms controlling the number of active rRNA genes during normal development.

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