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Evidence for Nucleolus Organizer Regions as the Units of Regulation in Nucleolar Dominance in *Arabidopsis thaliana* Interecotype Hybrids

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ABSTRACT

Nucleolar dominance describes the silencing of one parent's ribosomal RNA (rRNA) genes in a genetic hybrid. In *Arabidopsis thaliana*, rRNA genes are clustered in two nucleolus organizer regions, *NOR2* and *NOR4*. In F_8 recombinant inbreds (RI) of the *A. thaliana* ecotypes Ler and Cvi, lines that display strong nucleolar dominance inherited a specific combination of NORs, Cvi *NOR4* and Ler *NOR2*. These lines express almost all rRNA from Cvi *NOR4*. The reciprocal NOR genotype, Ler *NOR4*/Cvi *NOR2*, allowed for expression of rRNA genes from both NORs. Collectively, these data reveal that neither Cvi rRNA genes nor *NOR4* are always dominant. Furthermore, strong nucleolar dominance does not occur in every RI line inheriting Cvi *NOR4* and Ler *NOR2*, indicating stochastic effects or the involvement of other genes segregating in the RI mapping population. A partial explanation is provided by an unlinked locus, identified by QTL analysis, that displays an epistatic interaction with the NORs and affects the relative expression of *NOR4 vs. NOR2*. Collectively, the data indicate that nucleolar dominance is a complex trait in which NORs, rather than individual rRNA genes, are the likely units of regulation.

NUCLEOLAR dominance is an epigenetic phenomenon that describes nucleolus formation at the nucleolus organizer regions (NORs) inherited from only one parent of an interspecies (interspecific) hybrid (Reeder 1985; Pikaard 2000a,b; Viegas *et al.* 2002). NORs are chromosomal loci where ribosomal RNA (rRNA) genes are organized by the hundreds (sometimes thousands) in head-to-tail tandem arrays that span millions of base pairs (Ritossa and Spiegelman 1965; Wallace and Birnstiel 1966; Phillips *et al.* 1971). It is the transcription of rRNA genes by RNA polymerase I that brings about nucleolus formation (Nogi *et al.* 1991); thus the molecular basis for nucleolar dominance is the transcription of only one parental set of rRNA genes (Honjo and Reeder 1973; Chen and Pikaard 1997).

Nucleolar dominance could result from preferential activation of the dominant parental set of rRNA genes, preferential silencing of the underdominant set (note that the term "recessive" is inappropriate), or both. There is substantial evidence that enforcement (maintenance) of nucleolar dominance involves preferential gene silencing because underdominant rRNA genes can be derepressed by chemical inhibitors of DNA methylation (A. Viera *et al.* 1990; Neves *et al.* 1995; Chen and Pikaard 1997; Chen *et al.* 1998) or of histone deacetylation (Chen and Pikaard 1997). These data indicate that rRNA genes are kept silent by a mechanism that involves repressive chromatin modifications. However, the mechanisms that are initially responsible for choosing one progenitor's rRNA genes for inactivation and for establishing nucleolar dominance remain unknown.

In bread wheat, the addition of a single NOR-bearing chromosome from its wild relative, *Aegilops umbellulata* is sufficient to cause nucleolar dominance and to suppress the activity of the wheat NORs (Martini *et al.* 1982). This observation suggests that the presence of the dominant NOR alone may be sufficient to cause nucleolar dominance. Experimental results in Xenopus suggested that it is the rRNA genes themselves that are responsible for nucleolar dominance, presumably due to rRNA gene sequence differences that alter transcription factor binding affinities (Reeder and Roan 1984). According to this model, dominant rRNA genes are those with the highest affinity for one or more DNA-binding proteins that are available in limited supply relative to the number of rRNA genes (Reeder 1985). A correlation between nucleolar dominance and longer rRNA gene intergenic spacer length (which is where the promoter and other regulatory elements reside) in wheat was interpreted as being consistent with this model (Flavell 1986b).

Sequences other than rRNA genes located adjacent to the NORs have also been implicated in nucleolar dominance in flies. In Drosophila hybrids, chromosomal deletions in a heterochromatic region adjacent to the dominant NORs resulted in the loss of suppression at the underdominant NOR (Durica and Krider 1977, 1978). The latter observations can be interpreted as evidence

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that NORs might be regulated as single genetic elements, rather than as a collection of independently regulated rRNA genes, and may require regulatory sequences adjacent to the NORs.

The models outlined above share the idea that NORs, and possibly the rRNA genes within the NORs, possess the sequences that are responsible for uniparental rRNA gene expression in nucleolar dominance. However, genetic tests of the models have not been practical due to the sterility of interspecies diploid hybrids (*e.g.*, Xenopus, Drosophila) or the gene redundancy inherent to allopolyploid hybrids such as wheat.

We report here that nucleolar dominance occurs in fertile, diploid hybrids of the geographically isolated *Arabidopsis thaliana* ecotypes (natural strains or races) Landsberg erecta (Ler) and Cape Verde Islands (Cvi). Analysis of a Cvi/Ler recombinant inbred (RI) mapping population (Alonso-Blanco *et al.* 1998) shows that nearly complete nucleolar dominance can occur in lines homozygous for Cvi *NOR4* on chromosome 4 and for Ler *NOR2* on chromosome 2. However, not every individual inheriting this specific combination of NORs displays strong nucleolar dominance, indicating that the Cvi *NOR4*/Ler *NOR2* combination may be necessary, but is not sufficient, to explain nucleolar dominance. An unlinked locus identified by quantitative trait locus (QTL) analysis displays an epistatic interaction with the NORs and affects the relative expression of *NOR4 vs. NOR2*. These data indicate that modifier loci act on specific NORs and help determine the strength of nucleolar dominance.

MATERIALS AND METHODS

***A. thaliana* recombinant inbred lines and hybrids:** A collection of 162 recombinant inbred lines derived from reciprocal crosses of the *A. thaliana* ecotypes Ler and Cvi (Alonso-Blanco *et al.* 1998) was obtained from the Arabidopsis Biological Resource Center (ABRC; stock no. CS22000). The RI lines are descendants of 162 F_2 individuals that self-pollinated to yield F_3 seeds, one of which (for each line) was then grown and self-pollinated to yield F_4 seeds. The process of single-seed descent was continued to the F_8 generation. Cvi was the female in the cross leading to 118 of the RI lines (lines CVL1–CVL74 and CVL101–CVL147) and Ler was the female parent leading to 44 lines (CVL148–CVL193). The direction of the cross was found to have had no effect on the nucleolar dominance phenotype in RI lines. Genotypic data for molecular markers (49 on chromosome 1, 25 on chromosome 2, 41 on chromosome 3, 23 on chromosome 4, and 45 on chromosome 5) segregating among the 162 RI lines are available at http://www.dpw.wau.nl/natural/resources/populations/CVI/. The same Cvi and Ler stocks (Nottingham Arabidopsis Stock Centre stocks N8580 and N8581) used by Alonso-Blanco *et al.* (1998) to create the RI lines were used to make Cvi/Ler F_1 hybrids to examine nucleolar dominance in F_1 and F_2 generations. F_2 plants were grown from pooled seeds of multiple F_1 hybrid individuals.

Plant growth and nucleic acid extraction: Plants were grown for 3 weeks in a growth chamber (25°, 8 hr light, 16 hr dark). Three entire seedlings for each RI line, or one entire F_1 or F_2 seedling, were frozen in liquid nitrogen and ground to a powder. In a snap-cap tube, 3 vol (w/v) of extraction buffer (250 mm Tris-HCl pH 8.5, 375 mm NaCl, 25 mm EDTA pH 8.0, 1% SDS, 1% 2-mercaptoethanol) was mixed with the powder by vortexing. The homogenate was extracted with phenol/chloroform, and total nucleic acids were precipitated from the aqueous phase by addition of 1.5 vol of isopropanol. Following centrifugation, the pellet was resuspended in sterile water and total RNA was precipitated using 2 m LiCl. Genomic DNA was recovered from the resulting supernatant by ethanol precipitation. This procedure was performed twice for each RI line so that two independent tests of nucleolar dominance could be conducted.

Cleaved amplified polymorphic sequence (CAPS) analysis: A total of 100 ng of DNA from Cvi/Ler hybrids or RI lines was amplified by PCR in a 50-μl reaction containing 20 pmol each of forward primer 5′-agggggtgggtgttgaggga-3′ and reverse primer 5′-atctcggtatttcgtgcgcaagacg-3′ and 2.0 mm $MgCl_2$ and using 26 cycles of 94°, 30 sec; 65°, 30 sec; 72°, 40 sec. Resulting PCR products were digested with *Rsa*I (NEB) and subjected to agarose gel electrophoresis to reveal an ecotype-specific polymorphism.

Reverse transcription-CAPS analysis: RI lines with one Cvi NOR and one Ler NOR were tested for the presence of rRNA transcripts of each parental type using reverse transcription-CAPS (RT-CAPS). To do so, 1.0 μg of RNA, treated with RQ1 DNase I (Promega, Madison, WI) to eliminate contaminating DNA, was reverse transcribed using Superscript II (Invitrogen, San Diego) RNA-dependent DNA polymerase and an rRNA gene-specific reverse primer, 5′-atctcggtatttcgtgcgcaagacg-3′ in a 20-μl reaction using conditions recommended by the supplier (Invitrogen). A total of 1.0 μl of the reverse transcription reaction was then subjected to PCR and *Rsa*I digestion (CAPS analysis) as described above.

Quantitation of nucleolar dominance phenotypes for QTL mapping: A total of 10 μl of the RT-PCR reaction was digested with 10 units of *Rsa*I, resolved on a 2% agarose gel, and stained with ethidium bromide (RT-CAPS analysis). The resulting DNA bands in the gel were visualized using ultraviolet light transillumination and were recorded using a digital camera. Kodak ID 3.5.3 software was then used to determine the fluorescence intensity of Cvi- and Ler-specific bands. The fluorescence intensity of the Ler band was then divided by the intensity of the Cvi band and the resulting number was defined as the nucleolar dominance phenotype. In those cases in which no Ler transcript band was detectable, the numerical value 0.05 was assigned as the nucleolar dominance phenotype.

Statistical procedures: Single-marker analysis using JMP software (SAS Institute Version 5) was performed to locate potential QTL of large effect. Subsequently, a two-way ANOVA was performed to control for major QTL found in the single-marker analysis and to identify additional loci displaying epistatic interactions with these major QTL. The false discovery rate (FDR) was used to assess the significance of the results from both the single-marker analysis and the two-way ANOVA interaction test (Storey and Tibshirani 2003). The FDR is the rate at which features declared significant are truly null. For this analysis, FDR q-values were calculated for each P-value, with a q-value threshold of 0.05, meaning that 5% of the results declared significant could be null on average. The P-value, by contrast, is associated with the false-positive rate, the rate at which truly null features are declared significant. The FDR is considered the more appropriate criterion for genome-wide analyses (Storey and Tibshirani 2003). Chromosome-wide and genome-wide thresholds (calculated at $q = 0.05$, or 5%) were determined by this method. QTL scores above the chromosome-wide significance level are considered significant and scores above the genome-wide threshold are extremely sig-

nificant (Cheverud 2001; Lander and Kruglyak 1995). In addition, a randomized permutation test was performed using QTL Cartographer software to test the significance of QTL identified by single-marker analysis (Basten *et al.* 2002). In this test, nucleolar dominance phenotypes were randomly matched to genotypes 1000 times and significant ($P < 0.05$) outcomes that occurred by chance alone were compared to the results of the single-marker analysis (Churchill and Doerge 1994; Doerge and Churchill 1996). All single-marker QTL were significant above randomization, with the probability of false positives being <1:1000, consistent with the FDR tests.

RESULTS

Nucleolar dominance occurs within *A. thaliana*: Although transcribed regions of rRNA genes in *A. thaliana* ecotypes are almost identical (M. S. Lewis and C. S. Pikaard, unpublished results), a single nucleotide substitution that results in a *Rsa*I restriction site polymorphism made it feasible to distinguish rRNA transcripts of the Ler and Cvi ecotypes. The polymorphic *Rsa*I restriction site is located ~400 bp downstream of the transcription start site in Cvi rRNA genes (Figure 1A; the transcription start site is denoted as +1); this *Rsa*I site is absent in Ler rRNA genes. As a result, PCR of the region followed by *Rsa*I digestion (CAPS analysis) yields two bands for Cvi but only one for Ler (Figure 1A; compare lanes 1 and 2). In Cvi × Ler F_1 hybrids, each parental genome is equally represented and no recombination has taken place. Therefore, each F_1 hybrid has the same contribution of rRNA genes from each parent, as can be seen by comparing the DNA controls (denoted "D" in Figure 1B; lanes 1, 3, 5, 7). However, the Ler and Cvi rRNA genes are not equally transcribed in all F_1 individuals, as indicated by RT-CAPS analysis (denoted "R" in Figure 1B). For instance, in F_1 hybrids 1 and 2, Cvi transcripts are abundant, but Ler transcripts are detected in only trace amounts (lanes 2 and 4). By contrast, F_1 hybrids 3 and 4 display expression of both the Cvi and Ler rRNA genes (lanes 6 and 8), although Cvi transcripts are more abundant than Ler transcripts even in these individuals. These results show that nucleolar dominance occurs in a specific direction in interecotype hybrids of *A. thaliana*, but does so in a stochastic manner in the F_1. Stochastic onset of nucleolar dominance in newly formed hybrids, ranging from partial to complete dominance in F_1 hybrids, is not unprecedented, having been previously described in F_1 allotetraploid hybrids of *A. thaliana* and *Arabidopsis arenosa* (Chen *et al.* 1998).

In the F_2 generation of Cvi × Ler hybrids, following recombination between the two parental genomes and segregation of recombinant chromosomes, nucleolar dominance continued to be apparent among the F_2 population (Figure 1C). The dominance of Cvi over Ler rRNA gene transcription ranged from partial dominance (*e.g.*, lanes 2, 4, 12) to essentially complete dominance (lanes 6 and 16). In no case did an F_2 individual transcribe only Ler rRNA genes. The occurrence of nucleolar dominance among a segregating F_2 population suggested to us that the nucleolar dominance phenotype could be genetically mapped as a trait.

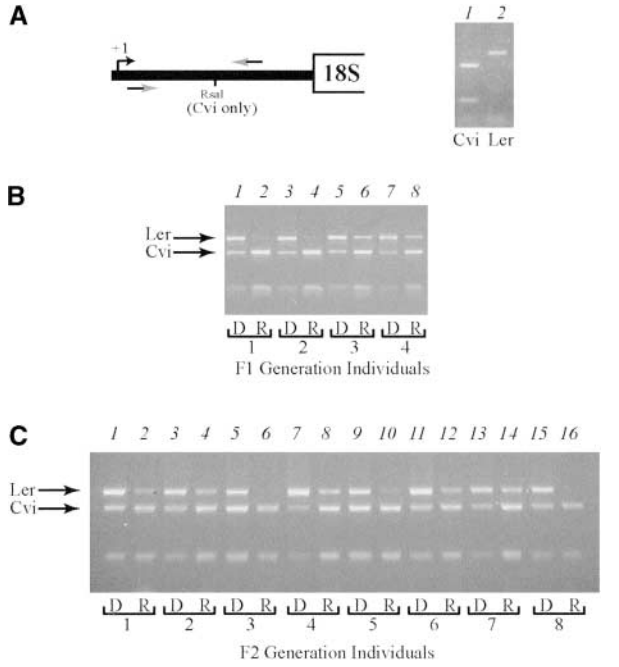

Figure 1.—Nucleolar dominance in interecotype hybrids of *A. thaliana*. (A) CAPS discrimination of rRNA genes of the ecotypes Cvi and Ler. In the region downstream of the transcription start site (+1), *Rsa*I cuts Cvi but not Ler rRNA genes. Locations of the primers used to amplify the region by PCR are denoted by arrows. Lanes 1 and 2 show PCR products of genomic DNA from Cvi and Ler, respectively, digested with *Rsa*I and subjected to agarose gel electrophoresis and ethidium bromide staining. (B) Four Cvi/Ler F_1 hybrids. Lanes 1, 3, 5, and 7 show *Rsa*I digestion of PCR-amplified genomic DNA whereas lanes 2, 4, 6, and 8 show *Rsa*I-digested RT-PCR products of RNA isolated from each individual. Note the detection of only Cvi rRNA transcripts in some F_1 hybrid individuals. (C) Eight Cvi/Ler F_2 hybrids. Odd-numbered lanes show *Rsa*I digestion of PCR-amplified genomic DNA from each individual whereas even-numbered lanes show *Rsa*I-digested RT-PCR products of RNA isolated from each individual.

Nucleolar dominance occurs in Cvi/Ler recombinant inbred lines: Recombinant inbreds have genomes that are a mosaic of sequences derived from two progenitors but are essentially homozygous at any given locus. We obtained a population of Ler/Cvi RI lines (F_8 generation) previously genotyped with respect to ~180 genetic markers (Alonso-Blanco *et al.* 1998). DNA from each RI line was tested by CAPS analysis as in Figure 1 to identify lines that contained both Ler and Cvi rRNA genes. Lines possessing both parental rRNA gene types in similar abundance and for which genotyping revealed a Cvi marker nearest one NOR and a Ler marker nearest the other NOR were chosen for further study (47 lines, listed in Table 1). In F_8 individuals, each locus is ex-

TABLE 1

Recombinant inbreds used in the study

RI line identifier	ABRC stock no.	Genotype at *NOR4*	Genotype at *NOR2*	Phenotype (Ler/Cvi)
CVL1	CS22001	Cvi	Ler	0.125
CVL4	CS22003	Cvi	Ler	1.250
CVL8	CS22007	Cvi	Ler	0.800
CVL15	CS22014	Cvi	Ler	0.600
CVL20	CS22019	Cvi	Ler	0.200
CVL22	CS22021	Cvi	Ler	0.325
CVL23	CS22022	Cvi	Ler	0.225
CVL24	CS22023	Ler	Cvi	1.250
CVL27	CS22026	Cvi	Ler	0.125
CVL33	CS22032	Ler	Cvi	1.700
CVL39	CS22038	Ler	Cvi	2.000
CVL43	CS22042	Ler	Cvi	0.600
CVL48	CS22047	Cvi	Ler	0.850
CVL53	CS22051	Cvi	Ler	0.050
CVL54	CS22052	Cvi	Ler	0.800
CVL58	CS22055	Cvi	Ler	0.950
CVL59	CS22056	Cvi	Ler	0.125
CVL70	CS22067	Cvi	Ler	0.075
CVL74	CS22071	Cvi	Ler	0.450
CVL101	CS22072	Cvi	Ler	0.150
CVL107	CS22078	Cvi	Ler	0.500
CVL108	CS22079	Ler	Cvi	1.500
CVL114	CS22085	Cvi	Ler	0.100
CVL115	CS22086	Cvi	Ler	0.125
CVL116	CS22087	Ler	Cvi	1.050
CVL117	CS22088	Cvi	Ler	0.550
CVL120	CS22091	Ler	Cvi	2.000
CVL124	CS22094	Ler	Cvi	1.000
CVL126	CS22096	Ler	Cvi	1.300
CVL127	CS22097	Cvi	Ler	0.400
CVL128	CS22098	Ler	Cvi	0.700
CVL137	CS22107	Ler	Cvi	1.750
CVL146	CS22116	Ler	Cvi	0.500
CVL154	CS22124	Ler	Cvi	1.050
CVL158	CS22128	Cvi	Ler	0.500
CVL163	CS22133	Ler	Cvi	0.600
CVL165	CS22135	Cvi	Ler	0.250
CVL166	CS22136	Ler	Cvi	1.350
CVL167	CS22137	Cvi	Ler	0.600
CVL169	CS22139	Cvi	Ler	0.300
CVL171	CS22141	Cvi	Ler	0.225
CVL172	CS22142	Ler	Cvi	0.450
CVL173	CS22143	Cvi	Ler	0.300
CVL180	CS22150	Ler	Cvi	2.100
CVL183	CS22152	Ler	Cvi	1.050
CVL191	CS22160	Cvi	Ler	0.075
CVL192	CS22161	Cvi	Ler	0.075

Recombinant inbred lines that possess both Cvi and Ler rRNA genes were used to identify QTL affecting nucleolar dominance. Original RI line designations and ABRC seed stock numbers are provided. Genotypes at *NOR2* and *NOR4* are based on inheritance of the nearest genetic markers, AD.156C and ANL2, respectively, and are consistent with CAPS analysis of each RI line. Nucleolar dominance phenotypes are the Ler/Cvi transcript ratios determined by RT-CAPS analysis. Values listed are the means of two trials, with each trial conducted using three individuals whose tissues were pooled to isolate RNA.

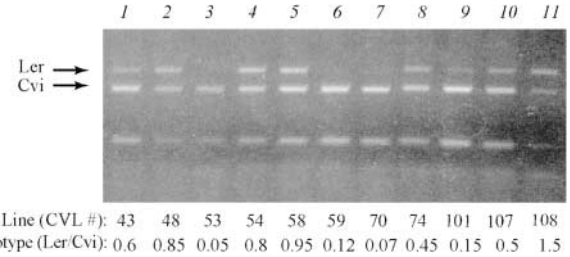

FIGURE 2.—Nucleolar dominance in Cvi/Ler recombinant inbred lines. *Rsa*I-digested RT-PCR products (RT-CAPS analysis) performed on a subset of the 47 RI lines found to possess one NOR inherited from Cvi and one NOR inherited from Ler (see also Table 1). Each RT-PCR reaction was conducted using RNA isolated from a pool of three individual seedlings. The nucleolar dominance phenotype is defined as the ratio of the intensities of the largest Ler and Cvi bands. The phenotype values listed are the mean of two independent trials of the RT-CAPS analysis but fit the data shown due to the reproducibility of the two trials.

pected to be homozygous (probability is 99.6%) such that lines containing rRNA genes of both parents are likely to have one NOR inherited from Ler and one NOR inherited from Cvi. This assumption is supported by the molecular marker data. All 47 RI lines found by CAPS analysis to contain both Ler and Cvi rRNA genes were also found to possess a Ler marker adjacent to one NOR and a Cvi marker nearest the other NOR (see Table 1). Because recombination between NORs and flanking markers is suppressed in Arabidopsis (COPENHAVER *et al.* 1998), as in other eukaryotes, the genotype at markers adjacent to the NORs is expected to represent the genotype at the NOR itself. Our CAPS data are consistent with this expectation. Our assignations of NOR genotypes in the remainder of this article are thus based on the genotypic and CAPS data and the assumption of no recombination between the NOR and its nearest flanking marker.

RT-CAPS analysis was performed on the selected 47 RI lines to determine their nucleolar dominance phenotype, defined as the Ler/Cvi largest band intensity ratio. This phenotype was determined for the 47 RI lines in two independent trials, with three seedlings of each RI line pooled to produce the RNA that was assayed in each trial. The two trials yielded highly reproducible results (89% repeatability determined by regression analysis). The mean value from these two trials was then calculated for each RI line. As summarized in Table 1, Ler/Cvi nucleolar dominance phenotype mean values ranged from 0.050 (the value assigned when only Cvi transcripts were detected) to 2.1 (Ler transcripts twofold more abundant than Cvi transcripts).

Plotting the nucleolar dominance phenotype relative to the NOR genotype (as well as visually inspecting the agarose gels) shows that nucleolar dominance is most pronounced in RI lines that have Cvi *NOR4* and Ler *NOR2* (Figure 3), resulting in a mean Ler/Cvi transcript ratio of 0.38. In other words, this specific combination

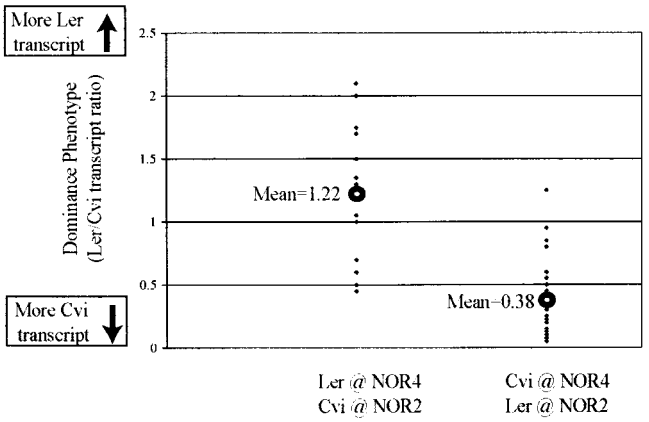

FIGURE 3.—RI lines with Cvi at *NOR4* and Ler at *NOR2* display a greater nucleolar dominance phenotype than RI lines with Ler at *NOR4* and Cvi at *NOR2*. Each diamond represents the mean phenotype (from two trials) for one RI line, and the circle represents the mean of the mean phenotypes for all lines having the specified NOR genotype. Note that when Ler contributes *NOR4*, transcript levels of Cvi and Ler tend to be similar, whereas when Cvi contributes *NOR4*, Cvi transcripts on average are ~3-fold more abundant than Ler transcripts and can be 20-fold greater or more (phenotypic values of 0.05).

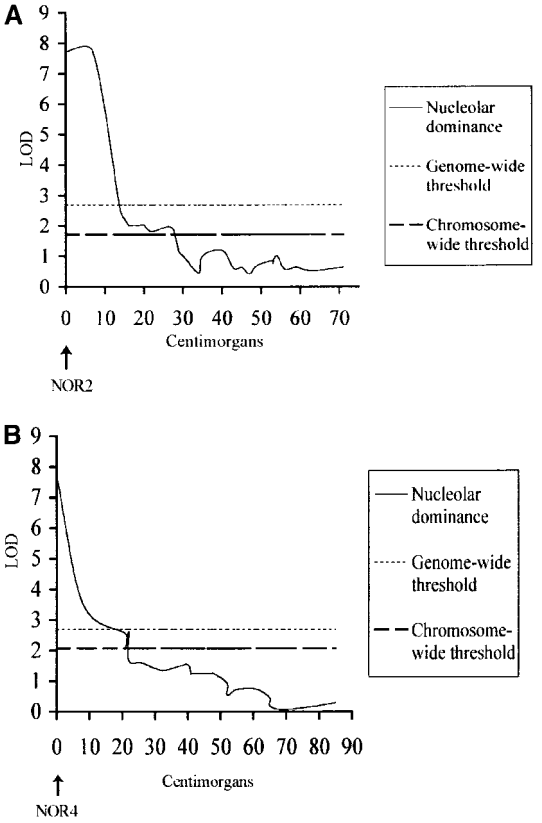

FIGURE 4.—Single-marker QTL analysis of nucleolar dominance in a Cvi/Ler recombinant inbred mapping population. The genetic distance on each chromosome is shown in centimorgans, with zero representing the junction of the NOR, which abuts the telomere, with centromere-proximal unique sequences. The LOD score represents the strength of the QTL. The thresholds represent a q-value of 0.05 calculated at the level of the genome and for each individual chromosome. The QTL at the NORs on chromosomes 2 (A) and 4 (B) are shown. It is expected that a QTL of equal intensity exists on both chromosomes because they are interdependent genetic loci in F_8 RI lines that were prescreened to find those that possess both Cvi and Ler rRNA genes. In other words, if one NOR is Cvi, the other is Ler, giving each associated QTL equal significance. No other chromosomes displayed significant QTL by single-marker analysis.

of NORs results, on average, in Ler rRNA transcripts being approximately threefold less abundant than Cvi transcripts. However, the range of phenotypes was substantial, with Cvi rRNA genes being completely dominant in some lines that inherited *NOR4* from Cvi and *NOR2* from Ler (Ler/Cvi transcript ratios of 0.15 or less, meaning a greater than sixfold dominance of Cvi over Ler; see Figure 2, lanes 3, 6, 7, 9) but not in other lines with this same NOR combination (see Table 1 and data points above the mean in Figure 3). By contrast, when *NOR4* was inherited from Ler and *NOR2* was derived from Cvi, the Ler and Cvi rRNA gene transcripts tended to be expressed at similar levels, with a mean nucleolar dominance phenotype (Ler/Cvi transcript ratio) that varied only twofold in either direction from a mean value of 1.22 (Figure 3).

QTL involved in nucleolar dominance include the NORs and an interacting locus: The variation in nucleolar dominance phenotype in RI lines that inherited Cvi *NOR4* and Ler *NOR2* suggested that other alleles segregating among the RI population might be needed in addition to this combination of NORs for nucleolar dominance to occur. Therefore, nucleolar dominance phenotypic values, combined with the data for the genotype markers segregating among the RI lines, were used to perform single-marker QTL analysis aimed at locating potential nucleolar dominance QTL of large effect. Genetic markers AD.156C and ANL2, nearest to *NOR2* and *NOR4* (at 0 cM on the genetic maps), respectively, and other linked markers were readily identified as major QTL, with the significance of the association decreasing with increasing distance from the NORs (Figure 4, A and B). It is important to realize that having one NOR from Ler in a highly inbred RI line with rRNA genes of both progenitors automatically means that the other NOR is Cvi. Thus the QTL associated with the NORs are mutually interdependent and cannot be distinguished from one another in these analyses. Therefore, we refer to these two QTL together as the NOR QTL. The NOR QTL account for only 51% of the variation seen in the nucleolar dominance phenotype (Table 2). The latter calculation provides a numerical explanation for the observation that inheritance of Cvi *NOR4* and Ler *NOR2* is associated with all cases of essentially complete nucleolar dominance yet does not guarantee its occurrence (see Figure 3 and Table 1).

Because the NORs were the only loci of strong effect

TABLE 2

QTL affecting nucleolar dominance

Locus	Chromosome	cM	LOD	Significance level	% variance
NORs	2/4	0/0	7.7	4.6×10^{-7}	51
GH.226C × NORs	3	23	2.6	0.0248	10
HH.158L × NORs	3	26	2.4	0.0248	9
EC.83C × NORs	3	27	2.5	0.0248	9
GD.318C × NORs	3	30	2.1	0.0352	8

QTL and their contribution to the variation in the nucleolar dominance phenotype. Chromosomal positions for the QTL are listed as are their LOD scores and significance levels. The significance level is the *q*-value calculated for the *P*-values determined by ANOVA and represents the likelihood that the QTL occurs by chance alone. The NOR QTL was determined by single-marker analysis. The remaining QTL, which map to a contiguous region of chromosome 3, are identified only in combination with the NOR QTL. Although GH.226C on chromosome 3 contributes the most variation to the phenotype, the entire region of chromosome 3 between 23 and 30 cM shows significant interaction with the NORs.

identified by single-marker QTL analysis, we looked for additional loci that contribute to nucleolar dominance but only in combination with the NORs. Therefore, a two-way ANOVA was performed using the NOR QTL marker ANL2 in combination with every other marker to find genetic interactions that contribute to nucleolar dominance. A significant epistatic interaction was found between the NOR QTL and several adjacent markers located on chromosome 3 (Figure 5A). This interaction accounts for as much as 10% of the variation seen in the nucleolar dominance phenotype and is strongest for marker GH.226C located 23 cM from the top of chromosome 3 (Figure 5A and Table 2). It is noteworthy that with relatively small sample sizes, there can be an upward bias in estimates of the percentage of variance explained by individual quantitative trait loci (Lynch and Walsh 1998). Therefore, it is possible that the percentages of variances reported here for QTL effects are higher than would be found had more than 47 RI lines bearing Cvi *NOR4* and Ler *NOR2* been tested. However, it is not likely that this bias leads to false-positive results.

Marker GH.226C shows an interesting pattern of interaction with the NORs, depending on the specific combinations in which the alleles occur in the RI lines (Figure 5B). Dominance of Cvi *NOR4* is most pronounced when the chromosome 3 QTL is inherited from Ler and is least pronounced when both *NOR4* and the chromosome 3 QTL are inherited from Cvi (Figure 5B). Interestingly, these data suggest that if Ler contributes the portion of chromosome 3 that corresponds to the QTL, the relative expression of *NOR4 vs. NOR2* is increased approximately twofold, regardless of whether *NOR4* is inherited from Cvi or Ler. By contrast, when the chromosome 3 QTL is inherited from Cvi, differences in the relative expression of *NOR4* and *NOR2* are less pronounced, causing Ler/Cvi transcript ratios to come closer to 1.0, which indicates codominance (Figure 5B).

DISCUSSION

Genetic analysis of nucleolar dominance has been hampered by limitations inherent to the use of sterile interspecific F_1 hybrids or allopolyploids as model systems. Our demonstration that uniparental rRNA gene expression occurs in fertile, diploid hybrids of geographically isolated ecotypes of *A. thaliana* provides a new system that circumvents these problems. In hybrids of the *A. thaliana* ecotypes Cvi and Ler, nucleolar dominance can be observed in the F_1 generation, as is the case for numerous interspecific hybrids that have been studied in the past. However, not every Cvi × Ler F_1 hybrid individual displays nucleolar dominance, indicating a stochastic aspect to the onset of nucleolar dominance. A stochastic onset to nucleolar dominance also has been observed in interspecific hybrids, specifically in synthetic allotetraploid hybrids of *A. thaliana* and *A. arenosa*, which recreate the natural allotetraploid species, *Arabidopsis suecica* (Chen *et al.* 1998). Allotetraploids maintain two copies of the NORs inherited from each progenitor yet express only the NORs of one progenitor. The recombinant inbred lines we have studied are homozygous for each NOR and thus they, too, have two copies of each progenitor's NORs in a diploid, yet in some cases (when Cvi contributes NOR4) express only one. On the basis of these considerations, we have no reason to suspect that uniparental rRNA gene expression in interecotype hybrids is different from nucleolar dominance as it has been studied traditionally in interspecies hybrids.

A long-standing question has been whether rRNA genes or complete NORs are the units of regulation in nucleolar dominance. Our analyses of nucleolar dominance in Cvi/Ler RI lines favor the latter hypothesis in that Cvi rRNA genes are not always dominant over Ler rRNA genes. Those RI lines that displayed essentially complete nucleolar dominance expressed Cvi rRNA

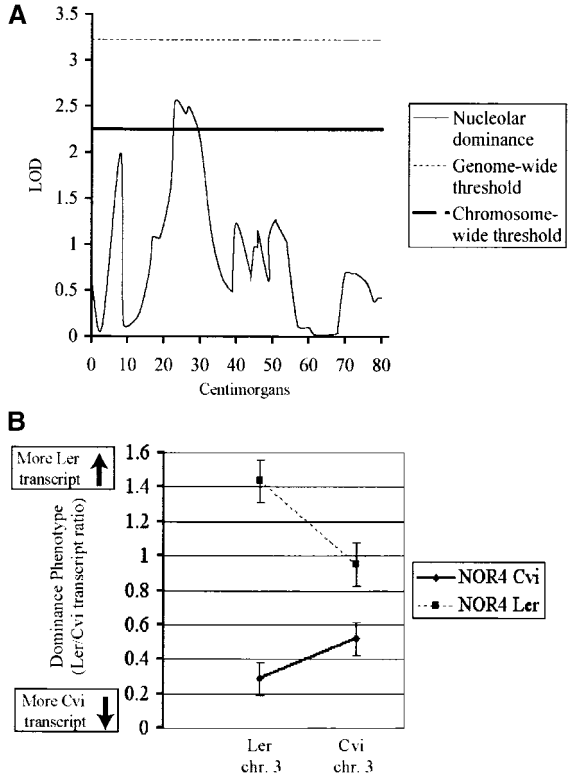

FIGURE 5.—A QTL on chromosome 3 interacts with the NOR QTL. (A) QTL analysis of genetic markers on chromosome 3 examining epistatic interactions with the NOR QTL. Genetic distance along the chromosome is given in centimorgans. The LOD score determines the significance of the interaction, with the thresholds set at $q = 0.05$ at the chromosome-wide or genome-wide level. (B) Allele-specific interactions between the NOR QTL and the chromosome 3 QTL. Data points show the mean dominance phenotype of the individuals possessing the markers indicated. Error bars indicate the standard error of the mean. The largest effect on nucleolar dominance is observed when Ler provides the chromosome 3 QTL. Note that expression of *NOR4* relative to *NOR2* is increased, regardless of parental NOR type, when the chromosome 3 QTL is inherited from Ler.

genes that had been inherited at *NOR4*. In these lines, transcripts from the Ler rRNA genes located at *NOR2* were undetectable or present in only trace amounts. Cvi rRNA genes were not strongly dominant when Cvi contributed *NOR2*. Given the well-known concerted evolution of rRNA genes, which causes their sequence homogenization within a species or population (DOVER and FLAVELL 1984; FLAVELL 1986a), one would expect the rRNA genes at Cvi *NOR2* and Cvi *NOR4* to be essentially identical in sequence. In keeping with this expectation, the *Rsa*I restriction site we used for CAPS analysis is absent in Ler rRNA genes but occurs in every Cvi rRNA gene and therefore must be present at both *NOR2* and *NOR4*. Furthermore, rRNA genes are >99% identical in sequence even between *A. thaliana* ecotypes, including Cvi and Ler, making it difficult to find sequence polymorphisms (COPENHAVER *et al.* 1995). A final argument is that some RI lines that inherited Cvi *NOR4* and Ler *NOR2* fail to display a strong nucleolar dominance phenotype. For these reasons, it seems highly unlikely that individual rRNA genes dictate the occurrence of nucleolar dominance.

The fact that all RI lines displaying a strong nucleolar dominance phenotype inherited Cvi *NOR4* and Ler *NOR2* suggests that there is something special about this specific combination of NORs. It is not simply the case that *NOR4* is always dominant and *NOR2* is always underdominant. For instance, when Ler rRNA genes make up *NOR4* and Cvi contributes *NOR2* the outcome is not strong dominance of Ler rRNA genes, but varying degrees of Ler and Cvi rRNA gene coexpression. *NOR2* and *NOR4* are known to be similar in size, at least in the Landsberg ecotype (COPENHAVER and PIKAARD 1996), suggesting that size variation in Landsberg NORs does not make *NOR2* more vulnerable to silencing. Although the sizes of Cvi NORs are not known, knowing this fact would not explain why only some, and not all, of the RI lines that inherit Cvi *NOR4* and Ler *NOR2* display a strong nucleolar dominance phenotype. Instead, it appears that unlinked modifiers, including the QTL identified on chromosome 3, affect the relative expression of *NOR4 vs. NOR2*, thus affecting the strength of the nucleolar dominance phenotype. Collectively, these data support the hypothesis that NORs are the units of regulation in nucleolar dominance.

Our results have parallels with previous studies in cereals that indicated a role for sequences unlinked to the NORs in regulating nucleolar dominance. In hybrids of wheat and rye, the wheat NORs are dominant and the rye NOR on the short arm of chromosome 1R is inactivated (NEVES *et al.* 1997a). However, translocation of the rye 1R short arm, including the NOR, onto the long arm of wheat chromosome 1 (1A, 1B, or 1D) results in codominance of the rye and wheat NORs (R. VIERA *et al.* 1990). Deletions or rearrangements in the long arm of rye chromosome 1R, the arm that does not include the NOR, also result in codominance of the rye and wheat NORs. Furthermore, substitution of rye chromosome 2R, which does not possess an NOR, by wheat chromosome 2D causes the rye NOR to be expressed (NEVES *et al.* 1997b). These observations indicate that unlinked sequences play roles in suppressing the rye NOR in a rye-wheat hybrid. Our ability to map at least one QTL unlinked to the NORs within a specific chromosome interval using the Arabidopsis interecotype hybrid system holds promise for ultimately identifying genes that act as modifiers of nucleolar dominance, which may be similar in Arabidopsis and cereals.

What might the QTL on chromosome 3 encode? The epistasis analysis (Figure 5B) suggests that the occurrence of Ler sequences at this locus causes an increase in *NOR4* rRNA gene transcription or a suppression of *NOR2* expression. Overexpression of an RNA polymerase I transcrip-

tion factor might cause increased transcription, although it is not clear why this would preferentially affect *NOR4*. Alternatively, nucleolar dominance is known to involve rRNA gene silencing due to cytosine hypermethylation and specific histone modifications (Chen and Pikaard 1997; Lawrence *et al.* 2004). The QTL on chromosome 3 could be a chromatin-modifying activity, although, again, it is not clear why repressive chromatin modifications would be preferentially targeted to *NOR2*. Nonetheless, it is intriguing that another study that used the Cvi/Ler RI population to map QTL affecting rRNA gene cytosine methylation identified a QTL on chromosome 3 (QTL3a) that overlaps the QTL identified in our study (Riddle and Richards 2002). The occurrence of Ler QTL3a was correlated with a significant increase in total rRNA gene methylation in those RI lines that also inherited Ler *NOR4* (note that *NOR2* could be either Cvi or Ler in their study). RI lines that had Cvi *NOR4* had equivalent total rRNA gene methylation levels regardless of whether Cvi or Ler contributed QTL3a. Had it been the case that the QTL3a identified by Riddle and Richards (2002) caused increased methylation when Ler *NOR2* was present, one could speculate that this increased methylation was preferentially targeted to *NOR2*, possibly causing decreased transcription at *NOR2* and thereby favoring increased *NOR4* expression. However, no such interaction between QTL3a and Ler *NOR2* was detected in their study. Thus it is not yet clear how the data of Riddle and Richards can help explain our results. Perhaps further insights could be gained if it were possible to discriminate between *NOR2* and *NOR4* of each ecotype in the methylation analyses and then see if *NOR2* were preferentially methylated when *NOR4* is inherited from Cvi and QTL3a is inherited from Ler. It is not clear, at present, how this could be done.

The QTL region on chromosome 3 includes a number of candidate genes that could potentially affect DNA or histone modifications involved in nucleolar dominance. Included in the region are a SET domain protein predicted to be a histone H3K9 methylase, as well as a DNA methyltransferase, a predicted methylcytosine-binding-domain protein, and an RPD3-like histone deacetylase. RPD3 is implicated in the conversion of active rRNA genes to an inactive chromatin configuration as cells enter stationary phase in *Saccharomyces cerevisiae* (Sandmeier *et al.* 2002). There are also four SWI/SNF genes in the region, which is interesting, given that a hSNF2 is a subunit of the nucleolar remodeling complex in mouse and human cells, a protein complex implicated in rRNA gene silencing (Santoro *et al.* 2002). One or more of these genes may influence the degree to which nucleolar dominance occurs, and further genetic dissection of the region will be needed to narrow the list of potential candidate genes.

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