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The Control of Natural Variation in Cytosine Methylation in Arabidopsis

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ABSTRACT

We explore the extent and sources of epigenetic variation in cytosine methylation in natural accessions of the flowering plant, *Arabidopsis thaliana*, by focusing on the methylation of the major rRNA gene repeats at the two nucleolus organizer regions (NOR). Our findings indicate that natural variation in NOR methylation results from a combination of genetic and epigenetic mechanisms. Genetic variation in rRNA gene copy number and *trans*-acting modifier loci account for some of the natural variation in NOR methylation. Our results also suggest that divergence and inheritance of epigenetic information, independent of changes in underlying nucleotide sequence, may play an important role in maintaining natural variation in cytosine methylation.

METHYLATION of cytosine is a common DNA modification widely distributed in both prokaryotic and eukaryotic kingdoms. Cytosine methylation is an important epigenetic mark that modifies the information content of the underlying genetic sequence. Perturbation of cytosine methylation, in mutants or by inhibitor treatment, leads to developmental defects in organisms ranging from plants and fungi to mammals (Li *et al.* 1992; Malagnac *et al.* 1997; Okano *et al.* 1999; Martienssen and Colot 2001). The phenotypic abnormalities are caused by the accumulation of genetic mutations (Chen *et al.* 1998; Miura *et al.* 2001) as well as heritable epigenetic alterations in DNA methylation (Kakutani *et al.* 1996; Jacobsen *et al.* 2000; Soppe *et al.* 2000) that lead to changes in gene expression. Cytosine methylation also interacts with other epigenetic modifications, such as histone deacetylation and histone methylation (Richards and Elgin 2002), to establish and maintain chromatin structures that influence transcription and recombination.

Both forward and reverse genetic approaches have identified important components of cytosine methylation systems in fungi, plants, and mammals. Common targets for reverse genetics are several different classes of cytosine DNA methyltransferases (Li *et al.* 1992; Finnegan *et al.* 1996; Ronemus *et al.* 1996; Okano *et al.* 1999; Bestor 2000), which add methyl groups to the five position of cytosine residues after DNA replication. Genetic screens have uncovered other *trans*-acting proteins necessary for maintenance of cytosine methylation, including a subset of SWI2/SNF2 proteins [Arabidopsis DDM1 (Vongs *et al.* 1993; Jeddeloh *et al.* 1998), mouse LSH (Dennis *et al.* 2001), and human ATRX (Gibbons *et al.* 2000)], and a histone H3 Lys9 methyltransferase [Neurospora DIM-5 (Tamaru and Selker 2001)]. In contrast, forward genetic screens have not identified *cis*-acting signals for cytosine methylation, although reverse genetic approaches have uncovered DNA sequence elements that may influence cytosine methylation levels (Yates *et al.* 1999; Miao *et al.* 2000). Despite this progress, the mechanisms that specify and maintain cytosine methylation patterns and levels remain poorly understood.

As an alternative to forward or reverse genetics, we exploited natural variation and quantitative trait locus (QTL) analysis to study the mechanisms by which cytosine methylation is controlled and inherited. The natural variation/QTL approach has the advantage of examining biologically relevant variation, including alleles of small effect, which have been maintained in natural populations. This strategy has the additional advantage of uncovering interactions between different loci involved in control of cytosine methylation. Genetic analysis using natural strains also provides the opportunity to examine the stability of different parental cytosine methylation patterns introduced into the same nucleus in hybrids.

We have chosen the model flowering plant, *Arabidopsis thaliana*, as a study system for this work, taking advantage of available natural accessions and genetic resources developed for this species (Alonso-Blanco and Koornneef 2000). We found significant natural variation in cytosine methylation, particularly in the arrays of rRNA genes [nucleolus organizer regions (NOR)], which constitute ~6% of the genome (Copenhaver and Pikaard 1996b). Our genetic analyses indicate that NOR cytosine methylation is controlled by a combination of factors, including *trans*-acting modifiers, variation in rRNA gene copy number, and epigenetic inheritance of parental methylation patterns.

MATERIALS AND METHODS

Plant materials: All plants were grown in a greenhouse or environmental chambers under standard growth conditions with 16 hr/day of light. The recombinant inbred (RI) lines

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used in this study as well as the natural accessions used in the survey were obtained from the Arabidopsis Biological Resource Center (ABRC) at The Ohio State University. The ABRC stock numbers are given in Table 1. The natural accessions were collected from different locations in Central Eurasia and North America (Alonso-Blanco and Koornneef 2000). In this study, single-seed descent lines derived from the natural collections were used, with the exception of the accession from Martuba (Mt-0; ABRC stock no. CS1380). Further, we took every natural accession line through at least two additional generations of single-seed descent starting from the seed aliquots from the ABRC. Because *A. thaliana* is predominantly self-fertilizing, these natural accessions correspond to independent genotypic variants.

Southern blot analysis: Leaf tissue was collected before flowering, and DNA was extracted from individuals as previously described (Jeddeloh *et al.* 1998). DNA for the initial Southern survey (Figure 1) was prepared from pooled tissue using QIAGEN (Valencia, CA) columns and protocols. Genomic DNA was digested according to the manufacturer's (New England Biolabs, Beverly, MA) recommendations. Southern blots were prepared and hybridized as described previously (Jeddeloh *et al.* 1998), using a radiolabeled fragment encompassing the 5.8S and 25S rRNA gene [pARR17 insert (Vongs *et al.* 1993), probe 1, Figure 1A]. NOR methylation levels were determined from phosphorimager (Bio-Rad, Richmond, CA) files using the Quantity One (Bio-Rad) software. The percentage of the hybridization signal in each lane above 1.0 kb corresponds to the percentage of methylation, based on the reference to the hybridization signal distribution in *ddm1* (strain Columbia), a mutant that demethylates the rRNA genes (Vongs *et al.* 1993; see Figure 1B). Methylation of the polymorphic intergenic spacer (IGS) region in Figure 5 was monitored using a hybridization probe corresponding to a 1.7-kb *Eco*RI fragment in the IGS (probe 2, Figure 1A). We investigated methylation at a single-copy genomic region using a 3.3-kb *Eco*RI fragment derived from λbAt105 (Pruitt and Meyerowitz 1986) as a hybridization probe. This DNA fragment encompasses a portion of the gene *MHC9.7*, the entire open reading frame of *MHC9.8*, as well as intergenic sequences (GenBank accession no. AP001305). The fragment itself includes three *Hpa*II sites; however, due to methylation at some of the neighboring *Hpa*II sites, up to eight different *Hpa*II sites are assayed by this probe.

rRNA gene copy number: rRNA gene copy number was determined using Southern blot analysis. Genomic DNA was digested with *Eco*RI (New England Biolabs), and Southern blots were prepared as described above. The blots were hybridized with a single-copy probe containing *MHC9.7* (AT3g21390) and *MHC9.8* (AT3g21400) and subsequently rehybridized with the pARR17 probe (probe 1, Figure 1A; Vongs *et al.* 1993). The hybridization signals were quantified using phosphorimager analysis (Bio-Rad), and the ratios between the rRNA gene signal and single-copy signal were calculated. An average of three measurements for each strain was determined, which was then normalized to the ratio for strain Columbia.

QTL analysis: The QTL Cartographer software suite was used to perform QTL analyses (Basten *et al.* 1994, 2001). The genotypic data for all the RI lines is available at http://nasc.nott.ac.uk. Single-marker analysis was followed by interval mapping according to Lander and Botstein (1989) using the Kosambi mapping function. In addition, we used a composite interval mapping approach that includes the most significant markers to control for genetic background effects. Our composite interval analysis used Zmap Model 6 in QTL Cartographer with a window size of 10 cM and 10 background markers (determined by stepwise regression in Srmap). Significance levels were set to 5% and estimated by performing 1000 permutations of each analysis (Doerge and Churchill 1996). Epistasis interactions between the five QTL identified were examined by a two-way ANOVA that included the single markers, as well as all two-way interactions possible. A second analysis to determine the amount of variation explained by each factor included only the significant two-way interactions between QTL. Heterozygotes in the RI population were treated as missing data in this analysis, which is presented in Table 2.

RESULTS

Using Southern blot analysis with methylation-sensitive restriction endonucleases, we examined 10 accessions of *A. thaliana*, collected from wild populations, for differences in cytosine methylation at both repetitive and low-copy genomic loci (Table 1). In all natural accessions examined, highly repetitive centromeric sequences, including the 180-bp *Hin*dIII repeats (Martinez-Zapater *et al.* 1986), the pericentromeric *Athila* retrotransposable elements (Pelissier *et al.* 1996), and 5S rRNA genes (Campell *et al.* 1992), were heavily methylated at *Hpa*II sites and showed no variation in DNA methylation (Table 1 and data not shown). We also found no variation in the methylation status of the single-copy *FWA* gene, which was previously shown to be a hotspot for epimutation (Soppe *et al.* 2000). However, we found significant variation in the methylation of two genomic regions in the 10 natural accessions: the single-copy *MHC9.7/9.8* locus on chromosome 3 (Pruitt and Meyerowitz 1986) and the major rRNA gene repeats at the end of chromosomes 2 and 4 (*NOR2* and *NOR4*; Copenhaver and Pikaard 1996a). At the *MHC9.7/9.8* region, we found a range of modification from complete methylation of all *Hpa*II sites examined in strain Ws to complete lack of methylation in strain Can (Table 1). There is no clear correlation between methylation at the *MHC9.7/9.8* locus and modification of the rRNA gene clusters (NOR; Table 1; Figure 1, A and B). NOR methylation ranged from ~20% in Can to >90% in Hi (defined by the percentage of total hybridization to *Hpa*II fragments >1 kb; see Figure 1B). However, even in natural accessions with comparable levels of NOR methylation, 5-methylcytosine appears to be differentially distributed along the major rRNA gene repeat. Natural accessions such as Hi show restriction fragments of intermediate size that are absent in other strains, such as Ler, despite similar levels of overall methylation (see Figure 1C). These intermediate-sized *Hpa*II fragments are most likely due to epigenetic variation in methylation patterns rather than to nucleotide sequence polymorphism because the rRNA gene repeat sequences are highly conserved among Arabidopsis strains. To examine the extent of variation of NOR methylation within strains, we quantitated NOR methylation levels from ~100 individuals for each natural accession. We found little intrastrain variation in NOR methylation, indicating that methylation of the major rRNA gene repeats is a stable characteristic of each natural accession (Table 1).

To study the basis of the observed variation in DNA

TABLE 1

Cytosine methylation content in different natural strains of Arabidopsis

Strain	Origin	ABRC stock no.	NOR %5mC[a]	Centromere	*MHC9.7/9.8*[b]
Can	Canary Island	CS6660	20.3 ± 0.4 (n = 72)	+	−
Col	Columbia	CS3126	75.2 ± 0.3 (n = 99)	+	±
Cvi	Cape Verde Islands	CS8580	28.9 ± 0.5 (n = 104)	+	±
Hi	Hilversum	CS6736	91.8 ± 0.2 (n = 88)	+	±
Kn	Kaunas	CS6762	36.0 ± 1.0 (n = 80)	+	+
Ler	Landsberg erecta	NA	70.9 ± 0.4 (n = 101)	+	±
Mt	Martuba	CS1380	26.4 ± 0.6 (n = 102)	+	±
No	Nossen	CS6805	50.2 ± 0.5 (n = 86)	+	±
Po[c]	Poppelsdorf	CS6839	43.0 ± 0.6 (n = 92)	+	−
Ws	Wassilewskija	CS6891	65.0 ± 0.3 (n = 93)	+	+
Zu	Zurich	NA	26.6 ± 0.4 (n = 78)	+	ND
	Cvi × Ler F_1 hybrid	NA	53.9 ± 1.5 (n = 3)	ND	ND
	Ler × Cvi F_1 hybrid	NA	57.9 ± 1.4 (n = 2)	ND	ND

[a] Average ± standard error (n, no. of individuals).

[b] Qualitative assessment of *Hpa*II site methylation at the *MHC9.7/9.8* locus: +, fully methylated; ±, partially methylated; −, no methylation; NA, not applicable; ND, not determined.

[c] Two types of individuals were present in this stock; the information refers to the low-methylation type.

methylation among *A. thaliana* natural accessions, we performed a QTL analysis. We measured NOR methylation in 162 recombinant inbred lines generated from a cross between a high NOR methylation parent (Ler) and a low NOR methylation parent (Cvi; Alonso-Blanco *et al.* 1998). Using single-marker analysis as well as interval and composite interval mapping, we identified five QTL that account for the majority of variation in NOR methylation between the parents (Figure 2). The major QTL, which together explain ~50% of the variation in NOR methylation (Table 2), map to the tip of chromosomes 2 and 4, where the *NOR2* and *NOR4* loci reside. The analysis also identified three *trans*-acting QTL, one each on chromosomes 1, 3, and 5, that explain ~20% of the NOR methylation variation between the Cvi and Ler parents (Table 2). We also calculated the effect of pairwise interactions between the five QTL and found three significant ($P < 0.1$) interactions involving four of the QTL (Table 2). The strongest interaction effect was between QTL3a and QTL4a (Figure 3).

The major QTL assignments to *NOR2* and *NOR4* in the Cvi/Ler cross indicate that the main determinants of NOR methylation are specified in *cis*. *Cis*-acting NOR methylation control could be explained by several mechanisms. One possibility is that the major rRNA gene repeats in Cvi and Ler have diverged at the primary DNA sequence level, which may lead to a difference in cytosine methylation. However, rRNA gene repeats are highly conserved, and sequence polymorphisms are rare in the transcribed region of the rRNA genes among *A. thaliana* natural accessions (C. Pikaard, personal communication; our unpublished results). Although the number of short tandem "SalI" repeats in the intergenic region can vary in different *A. thaliana* strains, leading to the formation of different intergenic spacer length variants (Copenhaver and Pikaard 1996b), there is no clear correlation between the variation in intergenic spacer length and NOR methylation (data not shown). These considerations argue against the hypothesis that sequence variation in the major rRNA gene repeats accounts for differences in cytosine methylation.

Another possibility is that differences in rRNA gene copy number affect NOR methylation levels. In most eukaryotes, rRNA genes are found in multiple copies and only a subset of these genes is expressed in most cells (Conconi *et al.* 1989; Dammann *et al.* 1993; Santoro and Grummt 2001). Strains with more rRNA genes may contain on average more transcriptionally quiescent gene copies, which can be subject to cytosine methylation associated with gene inactivity (Flavell *et al.* 1988). As shown in Figure 4, the relative copy number of 25S rRNA genes is positively correlated with NOR methylation in the 10 natural accessions surveyed. However, other factors must play a role as well. Strains with similar rRNA gene copy number, such as Cvi and Ler, can have markedly different levels of NOR methylation.

Epigenetic inheritance of parental methylation patterns is a third possible explanation for the strong QTL association with *NOR2* and *NOR4*. To test this hypothesis, we measured NOR methylation in F_1 hybrids between Cvi and Ler. 25S rRNA gene methylation in F_1 hybrids created by reciprocal crosses was intermediate between the two parental values (Table 1). This result could be due to strict inheritance of parental methylation patterns or a homogenization of methylation at intermediate values on all NOR in the hybrids. The latter hypothesis would be predicted if NOR methylation was set by an rRNA gene-counting mechanism acting on the whole nucleus level. We distinguished between these two mechanisms by examining the methylation of the two parental NOR types in the polymorphic intergenic region. Figure 5 shows that a polymorphic *Eco*RI fragment specific

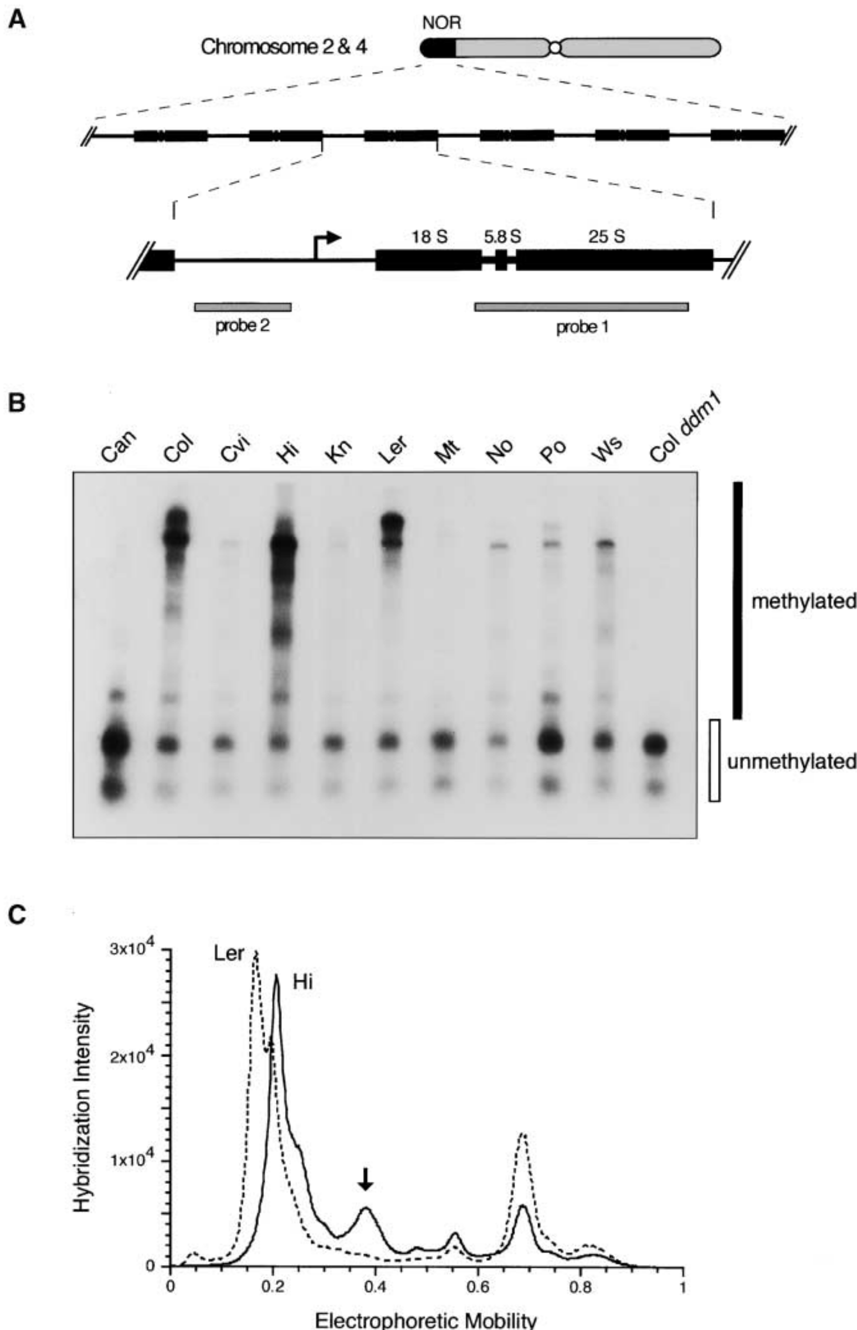

Figure 1.—Cytosine methylation of the Arabidopsis NOR. (A) The NOR at the upper tips of chromosomes 2 and 4 are composed of tandem arrays of a 10-kb repeat unit encoding the 18S, 5.8S, and 25S rRNAs. A bent arrow shows the location of the transcription start site, and the positions of the two hybridization probes used in this study are indicated below the monomer repeat unit. (B) Natural variation in NOR methylation. *Hpa*II-digested genomic DNA prepared from pooled tissue was size fractionated by gel electrophoresis and transferred to a nylon membrane. The membrane was hybridized with radiolabeled probe 1 (see A) and the signal was detected by autoradiography. Percentage of NOR methylation was calculated by measuring the percentage of hybridization signal in each lane (based on phosphorimaging) that corresponded to restriction fragments >1 kb. The 1-kb limit was chosen on the basis of the distribution of demethylated NOR hybridization signal seen in the Columbia *ddm1* mutant sample. (C) Qualitative differences in NOR hybridization signal (probe 1) between strains Hi and Ler are shown by comparing phosphorimaging intensity plots. The arrow indicates NOR restriction fragment or fragments of intermediate size present in Hi but absent in Ler.

for the low-methylation Cvi parent remains hypomethylated in the hybrids, as evidenced by complete digestion with *Hpa*II. These data indicate that parental NOR methylation patterns are maintained in Cvi/Ler hybrids.

DISCUSSION

Our survey of *A. thaliana* strains has uncovered significant natural variation in cytosine methylation restricted to certain genomic sequences. There is little variation among *A. thaliana* strains in terms of total cytosine methylation and most genomic regions examined show equivalent levels of methylation. In contrast, the different *A. thaliana* strains are highly variable in their cytosine methylation of the single-copy region encompassing predicted genes *MHC9.7* and *-9.8*. We found the most striking variation in cytosine methylation in the long arrays of rRNA genes found at the tips of chromosomes 2 and 4 (*NOR2* and *NOR4*). At the moment, we were unable to make any inference regarding the phenotypic effect of different NOR methylation levels due to the numerous genetic differences among the different *A. thaliana* strains.

After completing our survey of natural variation in cytosine methylation, we turned our attention to discerning the sources of this variation. Our investigation

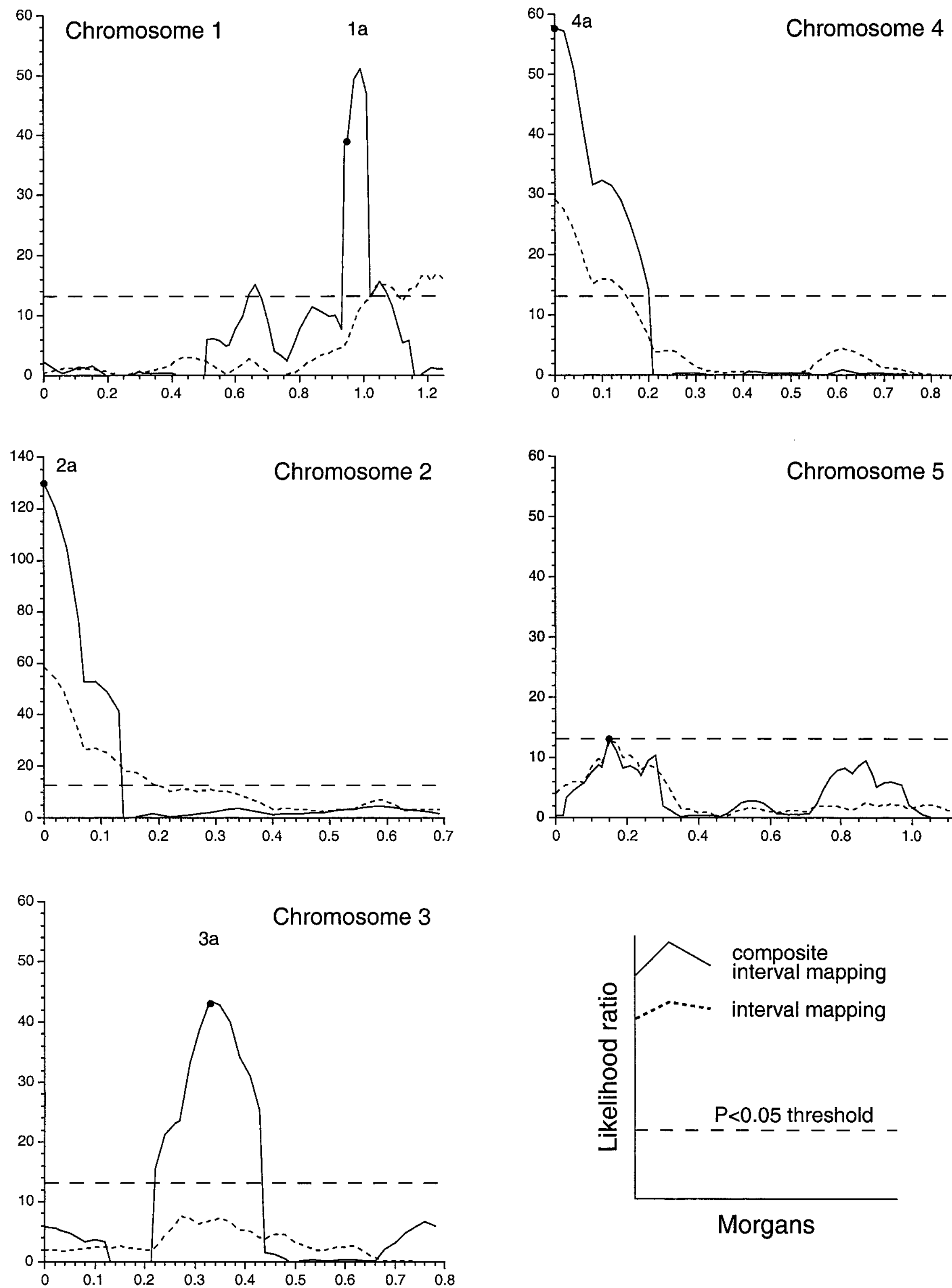

Figure 2.—QTL analysis of NOR methylation in a Cvi/Ler RI population. QTL associated with NOR methylation are mapped onto the five Arabidopsis chromosomes. Genetic distance on each chromosome is shown in morgans; 0 morgan is at the top of each linkage map. Strength of the association is reflected in the likelihood ratio (LR), which is directly related to the LOD score; LOD = 0.217 LR. The dashed line represents the 5% significance threshold calculated by a permutation test (1000 replicates). The dots indicate the position of the amplified fragment length polymorphism (AFLP) marker used in the ANOVA presented in Table 2.

TABLE 2

QTL and interaction effects

Effect	AFLP marker	F-ratio	P value	R^2
QTL 1a	CH.215L	25.8	<0.001	0.116
QTL 2a	AD.156C	155.3	<0.001	0.329
QTL 3a	AD.92L	39.6	<0.001	0.084
QTL 4a	ANL2	54.8	<0.001	0.167
QTL 5a	BH.325L	17.4	<0.001	0.030
QTL 4a × QTL 2a	NA	3.4	0.066	NA
QTL 3a × QTL 4a	NA	9.6	0.002	NA
QTL 3a × QTL 5a	NA	3.9	0.049	NA

F-ratios (d.f. = 1) and P values for the single QTL (nearest associated AFLP marker) were calculated by ANOVA including only the single markers and the significant interactions in the model. R^2 values correspond to the proportion of the variance explained by the individual QTL. NA, not applicable.

indicates that both genetic and epigenetic mechanisms lead to natural variation in cytosine methylation in Arabidopsis. One type of genetic variation that appears to specify NOR methylation content is rRNA gene copy number. The correlation between rRNA repeat number and cytosine methylation may relate to the general observation that highly reiterated sequences in eukaryotic genomes are often highly methylated. The mechanistic basis of the association between rRNA repeat number and cytosine methylation is not understood, but is consistent with the hypothesis that excess rRNA genes may be archived and transcriptionally silenced in association with increased cytosine methylation (Flavell *et al.* 1988). A simple gene-counting mechanism, however, cannot explain all of the natural variation in NOR methylation, because strains with similar rRNA gene copy numbers can possess widely divergent NOR methylation levels (*e.g.*, Cvi and Ler strains).

Some of the natural variation in cytosine methylation is likely to result from the genetic variation in genes encoding factors that direct and/or regulate DNA methylation. Our analysis suggests that genetic variation in such *trans*-acting modifier loci exists between the Cvi and Ler strains. Three *trans*-acting QTL affecting NOR methylation were identified in this study, mapping to

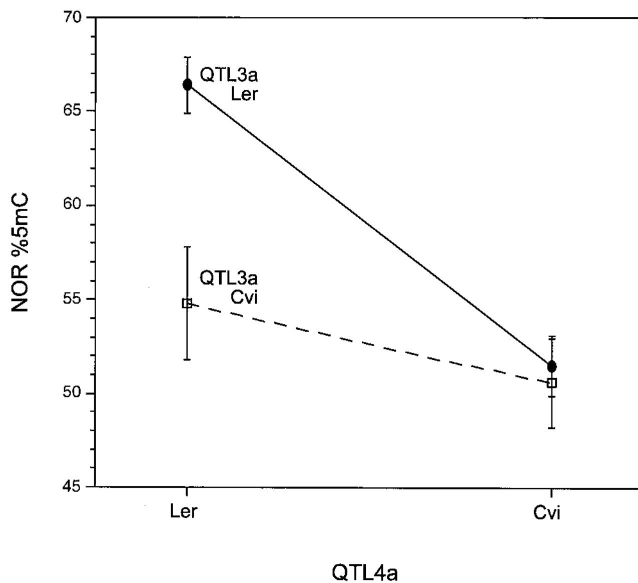

Figure 3.—Epistatic interaction between QTL3a and QTL4a. NOR cytosine methylation (means ± standard error) is plotted relative to QTL4a genotype. Solid line denotes this relationship in plants homozygous for the Ler allele at QTL3a; the dashed line plots the relationship in plants homozygous for the QTL3a Cvi allele. The difference in the slopes of the lines indicates that the genotype at one QTL influences the phenotypic effect of the other QTL.

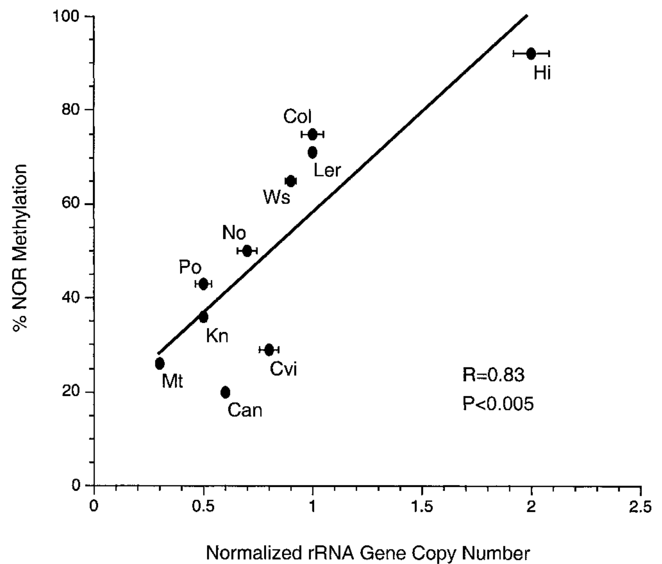

Figure 4.—Direct correlation between rRNA gene copy number and NOR methylation. The percentages of NOR methylation (Table 1) values were plotted against normalized rRNA gene copy number, which was determined by Southern analysis as described in materials and methods. Error bars represent standard error; in many cases, the standard error is too small to be visible. The line is a linear regression of the data (linear correlation coefficient, $R = 0.83$). Removal of the Hi data point changes the linear correlation coefficient to 0.79 ($P < 0.02$).

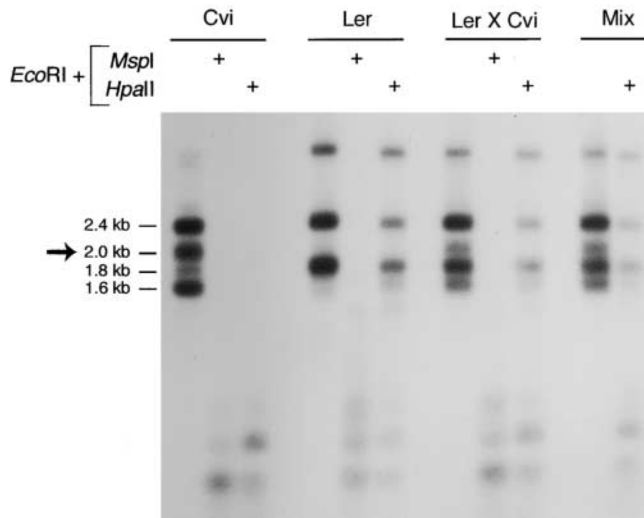

FIGURE 5.—Inheritance of the NOR hypomethylated state in Cvi/Ler hybrids. Genomic Southern analysis was used to examine the cytosine methylation state of the NOR intergenic region in individuals of the following genotypes: Cvi, Ler, and Ler × Cvi. As a control, a mixture of equal amounts of Cvi and Ler genomic DNA (Mix) was treated in parallel. Genomic DNA samples were digested with *Eco*RI, in combination with the methylation-sensitive *Hpa*II or its isochizomer, *Msp*I (which will cut C^{m}CGG). After transfer, the membrane was hybridized with a radiolabeled intergenic region fragment, probe 2 (see Figure 1A). The arrow indicates a hypomethylated 2.0-kb Cvi-specific *Eco*RI fragment from the intergenic region that remains hypomethylated in F_1 hybrids. The direction of the cross did not affect the results (data not shown).

chromosomes 1, 3, and 5. The identity of the genetic loci corresponding to these QTL is not known. We note that previously identified cytosine methylation modifiers, such as *DDM1* (Jeddeloh *et al.* 1998) and the cytosine methyltransferase gene *MET1* (Finnegan *et al.* 1996; Ronemus *et al.* 1996), fall outside of the windows encompassing the three *trans*-acting QTL. Both the Cvi and Ler parents must carry functional alleles for these established cytosine methylation modifiers because no relevant variation at these loci was segregating in the RI mapping populations examined. Of the 10 remaining predicted cytosine methyltransferase genes in Arabidopsis (http://www.chromdb.org), 3 of the predicted *de novo* cytosine methyltransferase genes in the *DOMAINS REARRANGED METHYLTRANSFERASE* class (Cao *et al.* 2000) fall within windows defined by QTL on chromosomes 3 and 5. *DRM3* maps close to the upper boundary of the QTL3a window, and *DRM1* and *DRM2* map in the vicinity of QTL5a. Additionally, the chromosome 5 QTL window corresponds to the genetic neighborhood containing the *KYP* histone H3 lysine 9 methyltransferase gene (At5g13960), which was recently shown to be important for specification of cytosine methylation at CpNpG sites (Jackson *et al.* 2002). Given the low resolution of QTL localization, these connections may be coincidental, but identification of such candidate genes may facilitate the large amount of work necessary to define the gene(s) corresponding to the *trans*-acting QTL.

The major QTL in our study map directly on the target loci, *NOR2* and *NOR4*. While several genetic mechanisms are consistent with this result, the finding that parental NOR methylation patterns are retained in Cvi/Ler F_1 hybrids (Table 1; Figure 5) points to epigenetic inheritance of diverged parental NOR methylation patterns as the simplest explanation for the major QTL. Transmission of differential NOR methylation patterns through meiosis contradicts the expectation in mammals, where cytosine methylation patterns are reset early in development (Monk *et al.* 1987; Reik *et al.* 2001). Our results, however, are corroborated by the inheritance of DNA methylation-based epialleles in plants (Jacobsen and Meyerowitz 1997; Cubas *et al.* 1999; Soppe *et al.* 2000; Knox and Ellis 2001). Further support for inheritance of parental cytosine methylation comes from the observation that *ddm1*-induced hypomethylation of genomic sequences is inherited in a Mendelian manner (Vongs *et al.* 1993; Kakutani *et al.* 1999).

It is not clear how parental cytosine methylation patterns are specified, but two general mechanisms can be envisioned. First, cytosine methylation patterns may serve as a template for maintaining identical or similar methylation patterns after DNA replication, as first proposed by Holliday and Riggs in 1975 (Holliday and Pugh 1975; Riggs 1975). This model requires that genomic sequences are not hypomethylated during early plant development; however, a report of a dramatic loss of cytosine methylation in tobacco pollen generative nuclei suggests that a DNA methylation erasure phase may exist in plants (Oakeley *et al.* 1997). An alternative model for inheritance of parental cytosine methylation patterns calls for specification by epigenetic marks independent of 5-methyl-cytosine. Such a model is consistent with the recent demonstration that methylation of histone H3 on lysine 9 is required for cytosine methylation in Neurospora (Tamaru and Selker 2001) and important for ^{m}CpNpG methylation in Arabidopsis (Jackson *et al.* 2002). Further clues to the mechanisms responsible may develop from study of situations in which the inheritance of parental methylation breaks down. The genetic interaction between QTL3a and *NOR4* (QTL4a) described here may represent such a situation. The genotype at QTL3a strongly influences the strength of QTL4a (Figure 3), suggesting that the inheritance of parental *NOR4* methylation patterns can be modified *in trans*.

While the inheritance of diverged DNA methylation patterns can contribute to the maintenance of natural epigenetic variation, a mechanism is still required to generate a divergent epigenetic state initially. Genetic variation at *trans*-acting modifier loci may play an important role. Indeed, the significance of *trans*-acting control of NOR methylation may have been underestimated in the present study because inheritance of widely divergent parental cytosine methylation patterns may overshadow

the influence of factors *in trans*. Epigenetic mechanisms may also contribute directly to the generation of divergent cytosine methylation states. Environmental conditions have been documented to alter cytosine methylation patterns in plants (Burn *et al.* 1993; Schmitt *et al.* 1997), and it is reasonable to propose that environmental stresses could lead to significantly divergent DNA methylation NOR haplotypes. Alternatively, small changes in cytosine methylation may accumulate over time, in a manner analogous to genetic drift of neutral genetic variation. We are currently testing both of these possibilities in an attempt to evaluate the role of epigenetic mechanisms in generating divergent cytosine methylation patterns.

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