DNA methylation and Lamarckian inheritance

By Hiroshi SANO

Research and Education Center for Genetic Information, Nara Institute of Science and Technology
Ikoma, Nara 630-0192
(Communicated by Yasuyuki YAMADA, M. J. A., Dec. 12, 2002)

Abstract: Jean Baptiste de Lamarck (1744-1829) maintained that characteristics that were acquired during an organism's lifetime are passed on to its offspring. This theory, known as Lamarckian inheritance, was later completely discredited. However, recent progress in epigenetics research suggests it needs to be reexamined in consideration of DNA methylation. In this article, I summarize our observations, which support Lamarckian inheritance. Initial experiments indicate that (1) artificially induced demethylation of rice genomic DNA results in heritable dwarfism, and (2) cold stress induces extensive demethylation in somatic cells of the maize root. Based on these results, I propose the hypothesis that traits that are acquired during plant growth are sometimes inherited by their progeny through persistent alteration of the DNA methylation status.

Key words: Acquired traits; DNA methylation; epigenetics; Lamarckian inheritance; vernalization.

Lamarckian inheritance. In 1809, Lamarck proposed that evolution is governed by two laws. First, organs are improved with repeated use and weakened by disuse. Second, such environmentally determined acquisition or loss of traits may be passed on to the offspring.[1] This theory was the first statement of inheritance of acquired characteristics, and became known as Lamarckism or Lamarckian inheritance. After intensive discussion, however, the theory was discredited by most geneticists after the 1930s.

Nevertheless, recent studies have raised the possibility that Lamarckian inheritance may play a partial role in development and evolution. This idea is based on the concept of "epigenetics", which is defined as change in gene expression without base sequence alteration.[2] This typically occurs during somatic cell differentiation in animal cells, in which the clonal expansion of a single cell leads to a diversity of cell types.[3] In other words, alteration of the genetic system within an organism is transmissible from cell to cell, this being called epimutation. Such cellular inheritance is common during ontogeny, but is usually erased when germ cells are formed.[4] This generally precludes inheritance of the epimutation by the next generation, and therefore cannot be considered to necessarily support the concept of Lamarckian inheritance.

In plants, however, it has long been known that epigenetically acquired traits can be sexually transmitted.[5] The best known example is flax (*Linum usitatissimum*). Plants treated with fertilizer exhibit a more branched pattern of growth and broader leaves than untreated plants.[6] Other characteristics are alteration in the plant weight at maturity,[6] the number of hairs on the seed-capsule septa[7] and the mobilities of isozymes of peroxidase and acid phosphatase.[8] These traits are in fact seed-transmissible.[6] In *Nicotiana rustica,* heritable differences have also been observed after a single generation of growth in specific environments.[9] One of the causes of such epigenetic inheritance is postulated to be the alteration of chromatin structure.[10] Consequently, the idea has been proposed that an environmental stimulus can induce heritable chromatin modification as an adaptive response, and therefore that inherited epigenetic changes in the structure of chromatin can cause a type of Lamarckian inheritance.[5]

DNA methylation. DNA of higher eukaryotes contains 5-methylcytosine (m^5C), comprising up to 30% of the total cytosines. In vertebrates, m^5C is located almost exclusively in CpG, while in plants it occurs in both CpG and CpNpG,[11] and also in non-symmetrical cytosines.[12] The distribution within the genome is nonrandom, and varies depending on the tissue and the

Table I. Characteristics of azadC-treated and untreated rice cultivated in the field

Year	Strain	Stem length (cm)	Panicle number	Panicle length (cm)	Grain number/ panicle	1000 grain weight (g)	Ear emergence (days)
1988	WT	109	nd	18.5	nd	26.6	146
	M_0	96	nd	16.0	nd	26.8	152
1989	WT	90	nd	17.4	nd	26.9	132
	M_1	74	nd	15.9	nd	25.4	137
1990	WT	109	14	22.6	134	24.9	nd
	M_2	82	21	23.8	110	24.1	nd
1991	WT	93	14	nd[a)]	nd	nd	91
	M_3	69	20	17.4	95	22.3	76
1992	WT	99	17	18.7	105	21.8	89
	M_4	80	17	18.6	101	19.8	78

Data fluctuation is due to different climate conditions during each year. Samples harvested in 1988 and 1989 are the original dwarf stock (M_0) and their offspring (M_1), respectively. Those harvested in 1990 (M_2) are stocks selected from the M_1 population to fix the dwarf trait. M_3 and M_4 are direct offspring of this selected M_2 generation. WT, wild-type; M_1 through M_4, mutated generations; nd, not determined. [a)]Not determined because of a typhoon which damaged grains before harvest. Standard deviations were estimated to be lower than 0.02.

developmental stage. The physiological function of m^5C is essentially to silence gene expression, which is important for host DNA defense against incorporation of parasitic DNA.[13] Intensive studies on animal cells have revealed distinct examples, including inactivation of the X-chromosome, imprinting of genes and silencing of parasitic DNA such as retroelements and transposons.[14] In plants, mobilization of transposons is also reported to be repressed by DNA methylation.[15]

The molecular mechanism of gene silencing is thought to involve changes in DNA structure caused by methylation of cytosines. Two possibilities have been proposed. One is direct repression due to methylated promoter regions blocking binding of transcriptional machinery, and the other is indirect repression due to altered nucleosome structures affecting the chromatin conformation.[13] Recent studies have indicated that the latter occurs frequently and plays an essential role in controlling global gene expression.[16] For example, various disorders including ICF (immunodeficiency, centromeric region instability and facial anomalies) have been found to be the result of abnormal chromosome structure caused by defective DNA methylation.[17] It is also known that chromosomal DNA is locally hypermethylated and globally hypomethylated in cancer cells.[18]

The controlling system of the on-off switch for DNA methylation, however, is still largely unclear. While a set of DNA methyltransferases has been identified in various organisms, including plants, the presence of DNA demethylases is controversial.[19] Eukaryotic DNA methyltransferases can be classified into two major groups.[20] One prefers hemi-methylated DNA, i.e. DNA with methylated cytosines on only one strand, and is possibly associated with the DNA replication complex functioning in maintenance of methylation patterns. The other methylates DNA on both strands, and establishes methylation patterns during embryonic development. These properties of two distinct enzymes make the DNA methylation patterns to be generally maintained through cell division, yet to be variable under certain circumstances. It is therefore believed that one of the factors responsible for epimutation is indeed methylation of DNA.[13]

Background observations. *DNA methylation in dwarf plants.* To determine whether or not plant growth is affected by DNA methylation status, the relationship between DNA methylation and dwarfism in maize (*Zea mays*) was initially examined.[21] The amount of m^5C in the DNA of a single-gene dwarf mutant, d5, was found to be about 8% lower than that of its tall, near-isogenic counterpart. To establish whether this DNA undermethylation was at all correlated with dwarfism, germinated seeds of a common tall strain (*Z. mays,* cultivar Honey Bantum) were treated for 16 h with 5-azacytidine (azaC), a powerful inhibitor of DNA methylation *in vivo.* After the chemical had been removed by repeated washing, seedlings were planted and the growth was measured. At maturity, the total stem length of treated plants was 28% less than that of untreated controls. Genomic DNA, isolated from azaC-treated plants, showed about an 8% reduction in m^5C content. No such effects were observed when seeds were

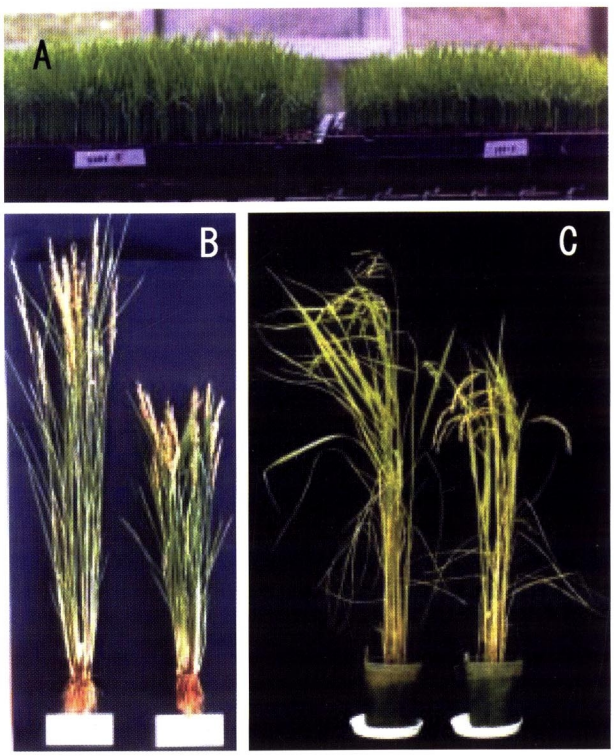

Fig. 1. Induction of heritable dwarfism in rice plants. Seeds of rice cultivars Akitakomachi (A and B) or Yamadanishiki (C) were imbibed for 3 days, treated with 0.1 mM azadC for 16 h, and cultivated under standard field conditions. Self-polinated offspring were successively obtained each year and tested for their traits. (A) Seedlings of M_3 generation (right) and control wild-type (left) of Akitakomachi. (B) Mature M_4 (right) and control wild-type (left) of Akitakomachi. (C) Mature M_5 (right) and control wild-type (left) of Yamadanishiki.

Table II. Frequency of m^5C in total cytosine residues in rice DNA[22]

DNA source	m^5C/total C (%)
Wild-type (Parent)	14.0
AzaC-induced dwarf (M_0)	11.7
Wild-type (F_1)	14.3
AzaC-induced dwarf (M_1)	12.0
AzaC-induced dwarf (M_2)	11.4

DNA was isolated from mature green leaves of 3-month-old plants. The ratio of m^5C to total C was calculated from experimental data by nearest neighbor analysis.

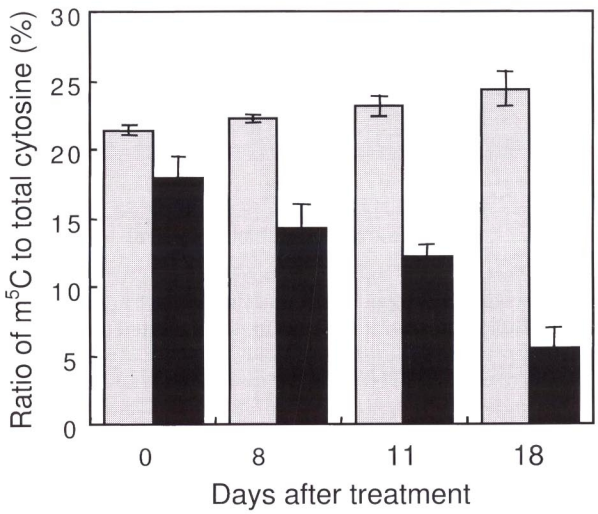

Fig. 2. Genome-wide demethylation in maize root tissues upon chilling.[24] The amount of m^5C in leaf blades (shaded) and root tissues (closed) was quantified by HPLC. DNA samples were extracted from 13-day-old seedlings grown at 23 °C (day 0); from seedlings that were further cold-treated at 4 °C for 8 days (day 8); and from seedlings returned to 23 °C for 3 days (day 11) or 10 days (day 18) after cold treatment. Note that cold-triggered demethylation proceeded even after samples were returned to the higher temperature. The ratio of m^5C to total C is expressed as a percentage (%).

treated with other nucleotide derivatives, such as 3'azido3'deoxythymidine and arabinosyl cytidine, indicating that the observed phenotypic and genomic alterations were azaC-specific. The results suggested that DNA methylation is indeed involved in the expression of genes regulating plant growth.

Induction of heritable dwarfism and demethylation. Because of its agronomic importance and the accessibility of good genetic materials, we further continued the project with rice (*Oryza sativa*) and analyzed the properties of azaC-induced dwarf plants in detail.[22] A single exposure of germinated rice seeds to either azaC or 5-azadeoxycytidine (azadC) induced dwarf plants. At maturity, seeds treated with these drugs showed normal morphological characteristics, except that their height (total stem length) was reduced by about 15% in comparison with untreated controls. The M_1 progeny, obtained by self-fertilization of azaC-induced dwarf plants, segregated into dwarf (35%) and tall types (65%). The M_2 offspring of self-fertilized dwarf M_1 plants were also dwarf, while those from tall M_1 plants were only tall (Table I). The heading time of these dwarf plants was 11 to 15 days earlier than with the controls. The dwarf and early heading properties were stably transmitted up to at least the M_5 generation (Fig. 1), whereas other characteristics, such as panicle length, number of panicles and total grain yield, remained the same as in the controls (Table I). The m^5C levels in genomic DNA isolated from mature leaves of wild-type tall, and azadC-induced dwarf plants were 14% and 12%, respectively, indicating about a 16% reduction in the m^5C content in dwarf samples. A similar reduction in m^5C content was also observed in the M_1 and M_2 progeny

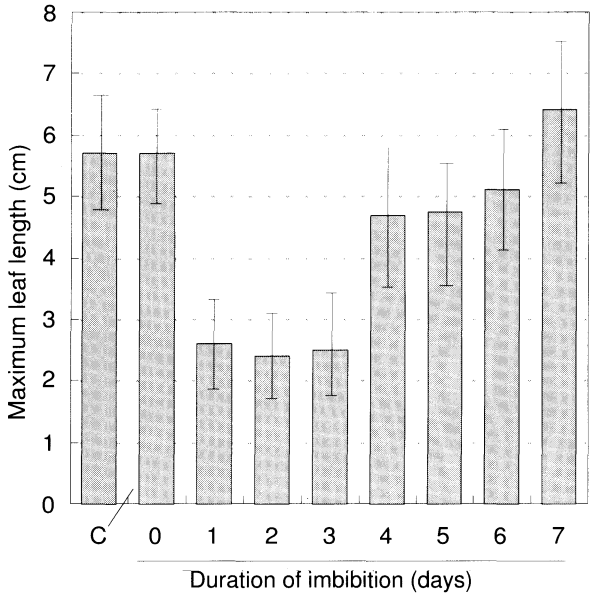

Fig. 3. Effects of azadC during seed imbibition. Approximately 100 dried seeds of wild-type Akitakomachi were set in a container with distilled water for imbibition for the indicated time period, and then treated with 0.1 mM azadC for 16 h. After washing repeatedly with distilled water, seeds were sown on soil and cultured under a light/dark cycle of 16 h/8 h at 23 °C in a plant incubator. Seedlings were allowed to grow for an additional two weeks, and plant height was measured. Control (C) was seedlings without azadC treatment.

(Table II). Thus, both hypomethylation and dwarfism induced by azaC treatment were heritable. The results suggest that azaC induced demethylation of genomic DNA, causing an altered pattern of gene expression and consequently a reduction in plant stem length.

Genome-wide demethylation and environmental stress. The above-described experiments clearly showed that artificial demethylation can induce heritable changes in the phenotype. The question then arises as to whether or not demethylation occurs under natural conditions, due for example environmental stress. To examine this possibility, we exposed maize seedlings to cold stress, and focused on whether the m^5C level changed.[23),24)] The results indicated that a genome-wide demethylation does occur in root tissues, but not in the above-ground parts (Fig. 2). Southern analyses also indicated a distinct reduction of methylation in Ac/Ds transposon loci in root tissues.[23)] Screening of genomic DNA identified one particular fragment that was demethylated during chilling.[24)] This 1.8 kb fragment, designated *ZmMI1*, contained part of the coding region of a putative protein and part of a retrotransposon-like sequence. *ZmMI1* was transcribed only under cold stress conditions. Direct methylation mapping revealed hypomethylated regions spanning 150 bases to alternate with hypermethylated regions spanning 50 bases. Analysis of nuclear DNA digested with micrococcal nuclease further indicated that these regions corresponded to nucleosome cores and linkers, respectively. Cold stress induced severe demethylation in core regions, but left the linker regions relatively intact. Thus, methylation and demethylation were found to be periodic in nucleosomes. Since DNA methylation in such sites induces alteration of gene expression by changing chromatin structure, extensive demethylation may serve as a common switch for many genes that are simultaneously controlled by environmental cues.

Demethylation of germ cells. Our experiments have so far indicated that acquired demethylation results in acquired traits, both of which are heritable for generations, and that demethylation occurs in somatic cells following environmental stress. The next question to be addressed is whether or not DNA methylation patterns in germ cells change in response to environmental stimulus. Currently, however, we have no clear evidence to indicate that this happens, although we have observed that germ cells can indeed be affected by artificial demethylation. Experiments were conducted to determine the most effective period for azadC treatment during rice seed cultivation. Seeds were subjected to imbibition for given periods to allow germination, then treated with azadC for 16 h, planted in soil and examined for plant height after two weeks (Fig. 3). The seeds were apparently susceptible to the drug if treated during the first 3 days of imbibition. If they were allowed to imbibe for more than 4 days, azadC treatment did not induce dwarfism. Estimation of the DNA methylation status then revealed that, when seeds were treated with azadC during the first 3 days of imbibition, the m^5C content decreased from 15% to as low as 2% (Table III). Seeds that were treated after more than 6 days' imbibition did not show any reduction in m^5C content. The majority of seedlings with reduced methylation, however, ultimately died after a month. Those survived grew normally to maturation, in which methylation nearly recovered the initial level (Table II). Since the observed dwarfism was heritable, these results suggested that germ cells, or cells that could develop into germ cells, are formed during the first 3 days of imbibition. This may imply that DNA methylation status does change in germ cells subjected to environmental pressure.

Table III. Effect of imbibition period on azadC-induced demethylation

DNA Source[a]	Percentage of m^5C in				
	CpA[c]	CpG	CpT	CpC	Total C
Untreated control	13.8±1.6	29.6±4.5	11.1±0.2	5.9±0.6	15.1±1.7
AzadC treated[b]					
0	4.8±0	4.5±0.2	5.0±0.5	2.3±0	4.2±0.2
2	2.7±0.7	2.5±0.2	2.2±0.4	1.5±0.5	2.2±0.5
3	4.4±0.3	3.9±0.3	3.9±0.2	2.1±0.3	3.6±0.3
6	12.6±0.6	26.1±1.7	11.2±0.4	5.8±1.6	13.9±1.1

[a]DNA was extracted from 2-week-old seedlings after azadC treatment.
[b]Samples were treated with azadC after the indicated imbibition period (days).
[c]Nearest neighbor analysis was performed as described.[22]

Hypothesis–inheritance of acquired traits through changes in DNA methylation. Based on the experimental data described above, I propose the following hypothesis: *A trait acquired by plants during growth in response to environmental stress is sometimes transmitted to the progeny through changes in DNA methylation status.* To provide evidence for this idea, several critical experiments are needed (Fig. 4). First, direct alteration of DNA methylation in germ cells upon external stimulus must be shown. Second, such an alteration must be correlated with alteration of phenotype. Third, expression of genes that are responsible for the phenotype must be shown to be regulated by DNA methylation status. Fourth, alteration of both DNA methylation pattern and phenotype must be heritable over several generations.

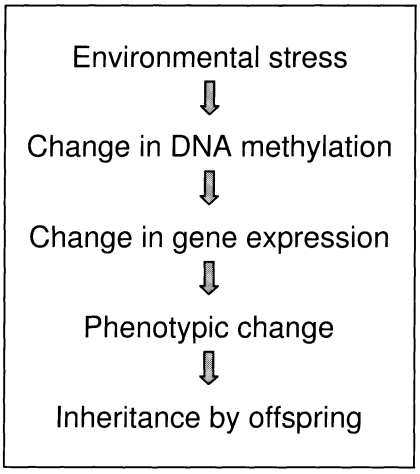

Fig. 4. Working hypothesis for inheritance of acquired traits. The critical point in this flow diagram is the alteration of DNA methylation in germ cells. It is also important to identify specific gene(s) that are involved in phenotypic change and whose expression is controlled by methylation.

The experiments may be best carried out using maize and/or rice, in both of which demethylation has already been shown to induce phenotypic alteration.[21),22)] The proposed experiments include approaches using physical stresses, including chilling and drought, instead of azadC. Although the epimutation frequency would be likely to be much lower than that caused by drug use, screening for stress tolerance would identify epimutated plants, whose methylation status could subsequently be analyzed. Further experiments would depend upon the results obtained, but gene screening and methylation assay methods are well established, making the procedures straightforward even though complicated and time-consuming. Overall, the entire series of envisaged experiments could be carried out with established methodology, and, if demethylation is proven to occur under natural conditions in the proposed system, the concept of Lamarckian inheritance, or epigenetic inheritance, will be substantiated.

Concluding remarks. Two other reports have so far described the possibility of epigenetic inheritance mediated by DNA methylation, one concerned inheritance of mouse color,[25)] and the other flower morphology in toadflax plants.[26)] Since inbred mice are genetically identical, they should show the same phenotype but coat color varied from yellow to mottled and was inherited from the mothers. Molecular analysis revealed a gene controlling coat color to be methylated, resulting in silencing, and that the methylated state was transferred from the mother through the germ line to her offspring.[25)] Although the mechanisms of methylation change in the mother are not clear, the results are clearly indicative of inheritance of an epigenetic modification. In the case of the toadflax (*Linaria vulgaris*), a mutant in which the fundamental symmetry of the flower is changed, was identified 250 years ago by Linnaeus. Recently, the gene responsible for flower development, *Lcyc,* was found to be heavily methylated and silenced in this mutant.[26)] Occasional revertants with a normal flower phenotype were shown to be demethy-

lated, and the *Lcyc* gene to be transcriptionally activated. It was thus concluded that heritable epigenetic mutations may play a significant role in evolution.

There are also observations that suggest a correlation between inheritance of epimutation and DNA methylation. An example is vernalization, which refers to a requirement of cold treatment for germination and/or flowering. Classically, the phenomenon is found in wheat, whose seeds can be planted in spring instead of in autumn provided that they were subjected to wetting and chilling during the winter. A recent example is a certain ecotype of *Arabidopsis*, which requires chilling prior to flowering. Since treatment with azaC caused early flowering in this plant, vernalization was proposed to be mediated by demethylation of the gene involved in flowering initiation.[27] Another example is cold-tolerance expression in wheat (*Triticum aestivum*).[28] Upon cold treatment, *Wcs120* genes, encoding stress responsive proteins, are transcriptionally activated. However, the extent of expression varies depending on the cultivar, being high in the strongly cold-tolerant cultivar, Cheyenne, but relatively low in the medium cold-tolerant cultivar, Chinese Spring. Common wheat is a hexaploid plant possessing three sets of the genome, A, B and D, and it was later found that the set of *Wcs120* genes in the A genome was inactive in Chinese Spring, while all of them were active in Cheyenne. The gene itself was intact in both cultivars, suggesting that a type of gene silencing, including methylation, was responsible for the inactivation.[28]

Taken the available information all together, I conclude that "Lamarckian inheritance" does exist, being mediated through DNA methylation. In line with this conclusion, I also suggest that the question of "Michurin biology"[29] concerning inheritance of acquired cold tolerance of winter wheat should be subjected to reexamination.

Acknowledgements. The author thanks Professor Emeritus Yasuyuki Yamada, M. J. A. (Nara Institute of Science and Technology) for the invitation to write this article, Professor Arthur B. Pardee and Dr. Ann B. Goodman (Harvard Medical School) and Professor Nam-Hai Chua (Rockfeller University) for valuable suggestions and discussion. The author also thanks Dr. Nicolas Steward, Dr. Ian Smith and Ms. Naoko Kishima (Nara Institute of Science and Technology) for experimental work, critical reading of the manuscript, and preparation of the manuscript, respectively. This work was supported by a grant of the Research for the Future Program (JSPS-RFTF 00L01604) from the Japan Society for the Promotion of Science (JSPS).

References

1) Lamarck, J. B. (1809) Philosophie Zoologique, Dentu, Paris.
2) Riggs, A. D., and Porter, T. N. (1996) In Epigenetic Mechanisms of Gene Regulation (eds. Russo, V. E. A., Martienssen, R. A., and Riggs, A. D.). Cold Spring Harbor Laboratory Press, New York, pp. 29-45.
3) Holliday, R. (1993) In DNA Methylation: Molecular Biology and Biological Significance (eds. Jost, J. P., and Saluz, H. P.). Birkhauser Verlag, Basel, pp. 452-468.
4) Reik, W., Dean, W., and Walter, J. (2001) Science **293**, 1089-1093.
5) Jablonka, E., and Lamb, M. J. (1989) J. Theor. Biol. **139**, 69-83.
6) Durrant, A. (1962) Heredity **17**, 27-61.
7) Durrant, A., and Nicholas, D. B. (1970) Heredity **25**, 513-527.
8) Cullis, C. A., and Kolodynska, K. (1975) Biochem. Genet. **13**, 686-697.
9) Hill, J. (1965) Nature **207**, 732-734.
10) Cullis, C. A. (1987) Amer. Naturalist **130**, S62-S73.
11) Gruenbaum, Y., Naveh-Many, T., Cedar, H., and Razin, A. (1981) Nature **292**, 860-862.
12) Oakeley, E. J., and Jost, J.-P. (1996) Plant Mol. Biol. **31**, 927-930.
13) Bird, A. P., and Wolffe, A. P. (1999) Cell **99**, 451-454.
14) Yoder, J. A., Walsh, C. P., and Bestor, T. H. (1997) Trends Genet. **13**, 335-340.
15) Miura, A., Yonebayashi, S., Watanabe, K., Toyama, T., Shimada, H., and Kakutani, T. (2001) Nature **411**, 212-214.
16) Bird, A. P. (2002) Genes & Devel. **16**, 6-21.
17) Robertson, K. D., and Wolffe, A. P. (2000) Nature Rev. Genet. **1**, 11-19.
18) Smith, S. S. (2000) J. Mol. Biol. **302**, 1-7.
19) Jones, P. A., and Takai, D. (2001) Science **293**, 1068-1070.
20) Finnegan, E. J., Genger, R. K., Peacock, W. J., and Dennis, E. S. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. **49**, 223-247.
21) Sano, H., Kamada, I., Youssefian, S., and Wabiko, H. (1989) Biochem. Biophys. Acta **1009**, 35-38.
22) Sano, H., Kamada, I., Youssefian, S., Katsumi, M., and Wabiko, H. (1990) Mol. Gen. Genet. **220**, 441-447.
23) Steward, N., Kusano, T., and Sano, H. (2000) Nucleic Acids Res. **28**, 3250-3259.
24) Steward, N., Ito, M., Yamaguchi, Y., Koizumi, N., and Sano, H. (2002) J. Biol. Chem. **277**, 37741-37746.
25) Morgan, H. D., Sutherland, H. G. E., Martin, D. I. K., and Whitelaw, E. (1999) Nature Genet. **23**, 314-318.
26) Cubas, P., Vincent, C., and Coen, E. (1999) Nature **401**, 157-161.
27) Burn, J. E., Bagnall, D. J., Metzger, J. D., Dennis, E. S., and Peacock, W. J. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 287-291.
28) Limin, A. E., Danyluk, J., Chauvin, L.-P., Fowler, D. B., and Sarhan, F. (1997) Mol. Gen. Genet. **253**, 720-727.
29) Hossfeld, U., and Olsson, L. (2002) Science **297**, 55-56.