Periodic DNA Methylation in Maize Nucleosomes and Demethylation by Environmental Stress*

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When maize seedlings were exposed to cold stress, a genome-wide demethylation occurred in root tissues. Screening of genomic DNA identified one particular fragment that was demethylated during chilling. 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. Direct methylation mapping revealed that hypomethylated regions spanning 150 bases alternated with hypermethylated regions spanning 50 bases. Analysis of nuclear DNA digested with micrococcal nuclease indicated that these regions corresponded to nucleosome cores and linkers, respectively. Cold stress induced severe demethylation in core regions but left linker regions relatively intact. Thus, methylation and demethylation were periodic in nucleosomes. The following biological significance is conceivable. First, because DNA methylation in nucleosomes induces alteration of gene expression by changing chromatin structures, vast demethylation may serve as a common switch for many genes that are simultaneously controlled upon environmental cues. Second, because artificial demethylation induces heritable changes in plant phenotype (Sano, H., Kamada, I., Youssefian, S., Katsumi, M., and Wabilko, H. (1990) Mol. Gen. Genet. 220, 441–447), altered DNA methylation may result in epigenetic inheritance, in which gene expression is modified without changing the nucleotide sequence.

DNA of higher eukaryotes is characterized by the presence of 5-methylcytosine (m5C)3 nucleotides, comprising up to 30% of the total cytosines. In vertebrates, m5C is located almost exclusively in CpG, whereas in plants it occurs in both CpG and CpgpG (1). The distribution within the genome is non-random and varies depending on the tissue and the developmental stages. The physiological function of m5C is essentially to silence gene expression, which is important for host DNA defenses against incorporation of “parasitic” DNA (2, 3). Two systems are involved: one directly blocks transcriptional machinery attached to promoter regions of genes by altering DNA structure and the other indirectly interferes with transcription by influencing nucleosome conformation and stability (4). Recent studies indicated the latter to occur frequently, as shown by various disorders due to abnormal chromosome structures that are caused by defective DNA methylation (5). One hallmark of cancer cells is local hypermethylation and global hypomethylation of chromosomal DNA (6). Abnormal methylation in the promoter regions of regulatory genes may indeed result in cancer development (7). Using antisense inhibition of DNMT1, a maintenance type DNA methyltransferase, about 10% of all genes in cultured mouse cells were found to be activated (8). These observations confirmed that DNA methylation functions as a global repressor of gene expression (9). The reverse case, i.e. global demethylation has also been inferred to be critical during embryogenesis in mammals (9, 10). What controls 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 (7). Among several candidate proteins, 5-methylcytosine DNA glycosylase was shown to induce genome-wide demethylation upon transfection into mouse myoblasts (11). Whatever the mechanism may be, reprogramming of DNA methylation appears to be fundamental in normal development (10).

Epigenetic inheritance is defined as change in gene expression without base sequence alteration (12). This typically occurs during somatic cell differentiation, in which the clonal expansion of a single cell leads to a diversity of cell types (13). Such a cellular inheritance is common during ontogeny but is usually erased before gametes are produced (14). In plants, however, it has long been known that epigenetically acquired traits can be sexually transmitted, as exemplified by flax (Linum) (15). In this case, epigenetic changes were induced by external factors such as nutrients and temperature (16, 17). Consequently, the idea has been proposed that an environmental stimulus can induce heritable chromatin modifications as an adaptive response (18). It is established that some clonal epigenetic changes are mediated through DNA methylation (6, 13), but evidence is limited for involvement of the latter in inheritance of acquired characteristics (19). We have previously shown that a single exposure of germinated rice seeds to the DNA demethylating agent, 5-azadeoxycytidine, induced dwarfism at maturity (20). Genomic DNA isolated from dwarf plants showed a 16% reduction in the m5C content in comparison with DNA from untreated plants. Both hypomethylation and dwarfism were transmitted to progeny for at least three generations (20). Thus, the acquired phenotype due to acquired changes in DNA methylation was heritable. However, whether or not such an epigenetic inheritance...
Demethylation of Maize DNA upon Cold Stress

Southern Hybridization, Genomic Library Screening, and Reverse Transcriptase-PCR—Genomic DNA samples from roots were digested with either MspI or HpaII (Takara), separated on agarose gels, and transferred to Hybond-N+ membranes (Amersham Biosciences). For library screening genomic DNA was digested with BanHI and fractionated on agarose gels. A 13-kb fragment was extracted and ligated to a zAP Express® BanHI/alkaline phosphatase-treated vector (Stratagene). Membranes were probed with a radioactively labeled 599-bp ZmMI1 fragment (GenBank®/EBI accession no. AF453523). For reverse transcriptase-PCR analysis total RNA was prepared by the uriniricarboxylic acid method (25) and treated with RNase-free DNase I (Takara). cDNA was then amplified by using an oligo-DT, as the primer and SuperScript® II (Invitrogen). cDNA was used as a template for ZmMI1 transcript-specific amplification (forward, 5'-GTCGGGGAG-GCTTCTTAAAGC-3' and reverse, 5'-CCCTAATGAGGCTCTGCTC-3') or actin transcript-specific amplification (forward, 5'-CGAAACAC-TGGTATGTGGATG-3' and reverse, 5'-TGTGGAAAATGTCGGAGA-GAG-3'). Samples were separated by electrophoresis on agarose gels and visualized with ethidium bromide staining.

Cytosine Methylation Mapping—Genomic DNA was digested with XbaI (Takara) and subjected to bisulfite modification (26). The modified DNA was subjected to PCR using ZmMI1-specific primers (forward, 5'-GAGGAA GAAGAAAAAGC-3' and reverse, 5'-AAATGT-CATTCTCTATTCTTATTC-3'). ExTaq enzyme (Takara), 250 μM dNTPs and 25 ng each dCTP, dGTP, dATP and dTTP were used for amplification on a PTC 210 thermocycler (MJ Research). PCR products were gel-purified and cloned into the plasmid pGEM-T Easy Vector® (Promega, Madison, WI), and cloned in JM109 (Stratagene, La Jolla, CA). The sequence was determined with an ABI PRISM BigDye® Terminator DNA sequencing kit and a 3100 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA).

Mononucleosomal DNA Assay—Roots from 13-day-old seedlings were harvested, and a pure preparation of nuclei was obtained by centrifugation on a 30% Percoll® cushion (Amersham Biosciences) (27). A 60-μg sample of nuclei suspension was digested with 4 units of micrococcal nuclease (Takara) at 30°C and fractionated on a 3.1 M sodium acetate/30% (v/v) ethanol solution (Vector Laboratories, Burlingame, CA). DAPI-stained nuclei were counted in a CytoFuge 4 CytoFuge (Cytomation, Inc., Salem, OR) and a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) was used to calculate the percentage of nuclei with chromatin condensation.

RESULTS

Genome-wide Demethylation upon Cold Stress—Analyses of maize DNA methyltransferase ZmMET1 during chilling (28) and of vernalization in Arabidopsis thaliana (29) revealed that cold stress might induce modification of the DNA methylation status. To obtain confirmatory evidence, we examined the level of total m5C in maize seedlings grown at 23°C for 13 days and then maintained at 4°C for up to 6 days. At the end of this treatment, growth had halted and tissue necrosis was apparent on leaf edges. Plants were therefore returned to normal culture at 23°C for an additional 7 days to allow recovery of frill growth, although the plants ultimately died after 2 weeks. Genomic DNA was extracted from leaf blades, stem mesocotyls, and root tissues at each step and subjected to nuclease P1 and phosphatase digestion to yield nucleosides, which were analyzed by high pressure liquid chromatography. The methylation level estimated from the ratio of m5C to cytosine was little

EXPERIMENTAL PROCEDURES

Plant Materials and Cold Treatments—Maize seeds (Zea mays L. cv. Golden Arrow) were germinated and hydroponically grown in a one-fifth strength Murashige and Skoog medium (Nihon Seiyaku, Tokyo, Japan) under continuous light for 13 days at 23°C and 70% relative humidity in a growth cabinet. Cold-pulse experiments were performed by transferring 13-day-old seedlings to an incubator at 4°C for 5 min. DNA was then isolated from each sample using a Prep-A-Gene Kit® (Bio101, Vista, CA), and the purity and concentration were determined by UV spectrophotometry. DNA was then treated with RNase A (Nacalai Tesque, Kyoto, Japan) at 37°C for 2 h to remove RNA contamination. After ethanol precipitation and denaturation at 100°C for 5 min, DNA was digested with 2 units of nuclease P1 (Sigma) at 37°C for 20 h, followed by dephosphorylation with 20 units of calf intestine alkaline phosphatase (TaKaRa, Otsu, Japan) at 37°C for 2 h. The sample was fractionated by Ultrafree-MC® PL-10 microcentrifuge tubes (Millipore, Bedford, MA), and the permeate was injected into a Superoxcel® LC-18-S column (Supelco, Bellefonte, PA). Separation was performed with a 2.5–20% methanol gradient in the presence of 50 mM KH2PO4, pH 4.3.

Fluorescent Immunostaining Histochemistry—Root tips were harvested from seedlings that were germinated and cultured at 23°C for 8 days in 70% humidity in a growth cabinet (AMC, Tokyo, Japan) or cultured at 24°C for 5 days following 5 days (cold-treated). Samples were fixed in an acetic acid: ethanol 1:3 solution for 16 h, rinsed in distilled water, and incubated for 30 min at 37°C in a digestion solution containing 4% cellulosomal RS® (Onozuka, Tokyo, Japan), 1% pectolysis Y-23, and 1 μM EDTA at pH 4.2 (21). Immunostaining were essentially performed as described (22) using an anti-5-methylcytosine monoclonal antibody (23) diluted in PBS containing 0.1% (w/v) Tween 20 and anti-mouse fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA) diluted 1:100 in PBST as the secondary antibody. Chromosomes were mounted in 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI)/ Vectashield® solution (Vector Laboratories, Burlingame, CA). Fluorescent images of DAPI and fluorescein isothiocyanate were observed by an AXC100 microscope equipped with UV excitation/activation and a charged-coupled device camera (Photometrics, Tucson, AZ), and visualized with the assistance of PhotoShop (ver. 5, Adobe, San Jose, CA).

Methylated CpG Island Screening—DNA samples were extracted from roots of control untreated seedlings and 6-day-old cold-treated seedlings as described above. Methylated CpG island amplification was performed by successive digestion of 5 μg of genomic DNA using Smal I and XmaI (24). Smal I cleaves unmethylated 5'-CCCGGG-3', giving blunt-end products, whereas XmaI cleaves the same sequence regardless of the methylation status, generating cohesive-end products. To prepare a cohesive-end adaptor, 50 μM each oligonucleotides 5'-CCCG-TAGCTAAGTGAACATT-3' and 5'-ATGCTAAGTGAACATT-3' were mixed in TE buffer and annealed at 65°C for 5 min. A 500-ng aliquot of digested DNA was then ligated to 1 pmol of adaptor with 400 units of T4 ligase (Takara) at 37°C for 16 h. This procedure yields only XmaI-digested DNA ligated to the adaptor. The resultant fragments were amplified by PCR with 30 cycles of 96°C for 20 s, 58°C for 25 s, and 72°C for 2 min using the single adaptor-specific primer 5'-ATGTTG-TCAATTGCTACCGGG-3', ExTaq® (Takara) and 250 μM dNTPs. Differential display screening was performed with an oligo-CTT, as the primer and SuperScript® II (Invitrogen). cDNA was used as a template for ZmMI1 transcript-specific amplification (forward, 5'-GTCGGGGAG-GCTTCTTAAAGC-3' and reverse, 5'-CCCTAATGAGGCTCTGCTC-3'). The optimal cycle number was determined to ensure an exponential range of amplification, and the loading amount of amplified DNA to agarose gel was selected to ensure a linear range of signal intensity after ethidium bromide staining. Amplified DNA was quantified by densitometric Image Gauge V3.3 software (Fuji Film Science Laboratory and Kohshin Graphic Systems, Tokyo, Japan).
affected in leaf blades and stem mesocotyls, m$^5$C accounting for ~20% of the total cytosines throughout the treatment period (Fig. 1, A and B). In contrast, the methylation level clearly decreased in roots upon cold treatment (Fig. 1 C). A notable feature was that, even after seedlings were returned to normal growth conditions, the decreased methylation level did not recover but rather continued to decline, reaching one third of the original level (Fig. 1 C). The global status of methylation and its alteration upon cold treatment was histochemically examined in 8-day-old seedlings using antibodies against m$^5$C. The distribution of m$^5$C was not random in and among chromosomes (Fig. 2). In a chromosome, heavily methylated regions alternated with undermethylated regions. Some chromosomes were less methylated than others (Fig. 2). A hypomethylated region in chromosome 4 was assigned to the knob, which consists of TR-1 repeats forming fold-back DNA segments (30). However, no distinct difference was observed in methylation patterns among chromosomes from untreated and cold-treated seedlings (data not shown), probably due to the inability of antibodies to provide quantitative analysis under the experimental condition.

**Isolation and Characterization of Demethylated Genomic Sequences**—To identify specific sequences demethylated in root tissues upon cold stress, methylated CpG island amplification (24) and PCR differential display screening were performed. Initial screening identified nine genomic fragments that were possibly demethylated on chilling (data not shown), one of which (registered as GenBank®/EBI accession no. AF453522) was further used as the probe and analyzed by Southern blot hybridization using MspI and HapII m$^5$C-sensitive restriction enzymes. An ~0.6-kb fragment was distinct in DNA from cold-treated seedlings, whereas it was absent in DNA from untreated seedlings (Fig. 3A). This indicated two cytosines in CCGG to be methylated in controls but demethylated in chilled samples, this being confirmed later by direct methylation mapping sequencing (data not shown). The isolated fragment was used as the probe to screen a genomic DNA library, and a 1756-bp fragment was finally isolated (registered as GenBank®/EBI accession no. AF453522) (Fig. 3B). This genomic fragment, designated as ZmMI1, showed a similarity at the 3’-region from position ~1400 with the PREM-1 long terminal repeat (registered as GenBank®/EBI accession no. U03681), a putative retroelement (Fig. 3B). A part of common sequences between 480 and 800 matched well to a maize 348-bp expressed sequence tag clone, which was induced upon salt stress to seedlings (registered as GenBank®/EBI accession no. AI967092) (Fig. 3B). The copy number of ZmMI1 was estimated to be ~40–50 by Southern blot signal intensity analysis (data not shown). To identify possible transcription of this sequence,
a cDNA library was screened and six clones were obtained. All of the sequences, however, slightly differed each other, probably reflecting transcription from different loci (registered as GenBank®/EBI accession nos. AF468668 through AF468672). The position of poly(A) addition was around 1100 (Fig. 3C). When cold-treated samples were returned to 23°C with additional 4 days (closed bar), the number of examined clones were 64 for control (A), 75 for cold-treated (B) and 34 for cold-chased (C) samples. Position of C in ZmMI1 genomic sequence is indicated on the horizontal axis and frequency of m^5C in the C at the indicated position is expressed on the vertical axis (%). The average methylation frequencies were 38.2% in control (A), 24.7% in cold-treated (B) and 22.5% in cold-chased (C) samples. (D) Global distribution and proportion of m^5C among cytosine-containing motifs in the mapped 886 bases of ZmMI1 sequence in seedlings grown at 23°C (open bar), cold-treated at 4°C for 7 days (shaded bar) and returned to 23°C with additional 4 days (closed bar). Numbers of cytosine-containing motifs in the mapped sequence were 83 for CpG, 68 for CpNpG, 121 for others (orphan C) in total 255 cytosines. Values for methylated motifs are indicated as percentages (%).
preferentially in the /H11011D
some methylation sites from 75% to 5%. The
phoresis. A 200-bp fragment indicated by the
and resulting DNA fragments were subjected to agarose gel electro-
micrococcal nuclease digestion, fragments containing hypomethylated regions were
nucleosomal DNA fraction. Intact chromatin extracted from isolated
maize seedlings nuclei was subjected to micrococcal nuclease digestion,
nucleosomal DNA fraction. Intact chromatin extracted from isolated
is composed of a unit of six nucleosomes. Linker regions are
hence corresponded to the linker regions. We concluded there-
DNA in the core and linker regions of nucleosomes was hypo-
and hypermethylated, respectively. However, whether or not such differential methylation patterns corre-
with methylation frequency on the /H11011A
maximized from hypermethylated regions and
fragments were isolated (Fig. 5B). When genomic DNA was
or hypomethylated regions (Fig. 5B). When genomic DNA was
or passive mechanisms have been proposed (11, 36). In active demethylation,
—dependent on feed-back loops between modification of chroma-
Methylation of histones was shown to be essential for
structures, which in turn proteins and DNA (35). Our result showing periodic oscil-
histone H1 to methylated DNA have been reported (32, 33), and cur-
ucellular loss of DNA methylation occurs, for which active or passive mecha-
ubiquitous demethylation at CpG, CpNpG and other sites, sug-
DNA methylation in filamentous fungi, and it was
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delines between methylated DNA and proteins that constitute chroma-
static interaction between methylated DNA and proteins that constitute chroma-
hypermethylated cytosines were sensitive to nuclease and hence corresponded to the linker regions. We concluded there-
whether or not such differential methylation patterns correlated with the mode of nuclease digestion was not clear from the
that DNA in the core and linker regions of nucleosomes
in untreated seedlings is illustrated after Fig. 4A. Fragments 1, 3, and
of obtained values for a 200-bp DNA template to those for genomic DNA
ubiquitous demethylation at CpG, CpNpG and other sites, sug-
and subjected to digestion with micrococcal nuclease, which
amount of each fragment was densito-
periodic oscillation pattern in every 200 bases
B
and subjected to agarose gel electrophoresis and visualized
maize seedlings nuclei was subjected to micrococcal nuclease digestion,
resulting DNA fragments were subjected to agarose gel electro-
be responsible for periodic methylation and preferential
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mC, but the experimental evidence is controversial. For example, opposite results as to preferential binding of histone
another distinct finding of the direct methylation mapping is
is consistent with this view and further suggests a positive role
methylation within the nucleosomal structure is
currently the interaction between histones and methylated DNA is
in general not considered to be specific. However, under certain circum-
Demethylation of maize DNA upon cold stress
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in untreated seedlings is illustrated after Fig. 4A. Fragments 1, 3, and
of obtained values for a 200-bp DNA template to those for genomic DNA
taken as 100%) was determined. Numbers correspond to those illustrated in B.
was hypo- and hypermethylated, respectively. However,
while two distinct fragments were equally well amplified (Fig. 5C).
Demethylation of CpG was more distinct than that
sequence, some factor(s) independent of sequence may
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demethylation is catalyzed by 5-methylcytosine DNA glycosidase
in plants. A specific feature of our findings was that demethylation
revealed by direct methylation mapping. A question arises,
and preferential demethylation.
Association of the Methylation Pattern with the Nucleosomal Structure—The periodic oscillation pattern in every 200 bases
suggests an association with the nucleosome structure. To assess this possibility, root nuclei were extracted from seedlings
and subjected to digestion with micrococcal nuclease, which preferentially cleaves chromatin DNA in the spacer regions.
Resultant ~200-base fragments were isolated (Fig. 5A) and subjected to PCR in parallel with control undigested genomic
DNA. Primers were designed to independently amplify five
~150-base fragments, each containing either hypermethylated
or hypomethylated regions (Fig. 5B). When genomic DNA was
used as the template for PCR with five sets of primers, corre-
spending fragments were equally well amplified (Fig. 5C).
When nuclease-digested 200-base DNA fragments were em-
ployed, fragments containing hypomethylated regions were
more efficiently amplified than those containing hypermethyl-
ated regions (Fig. 5C). This suggested that micrococcal nuc-
lease preferentially cleaved DNA around the hypermethylated
regions. In other words, ~145-base regions enriched with hy-
pomethylated cytosines were resistant to nuclease and hence corresponded to the core, whereas ~45-base regions enriched
with hypermethylated cytosines were sensitive to nuclease and hence corresponded to the linker regions. We concluded there-
that DNA in the core and linker regions of nucleosomes
was hypo- and hypermethylated, respectively. However,
whether or not such differential methylation patterns correlated with the mode of nuclease digestion was not clear from the
present finding.

DISCUSSION
Nucleosomes are organized into precise positions on DNA by signals in the sequence (31). One such signal has been proposed to be mC, but the experimental evidence is controversial. For example, opposite results as to preferential binding of histone
H1 to methylated DNA have been reported (32, 33), and cur-
cently the interaction between histones and methylated DNA is
in general not considered to be specific. However, under certain circum-
stances, nucleosome assembly appears to depend on CpG methylation (31, 34). This suggests a dynamic interaction
between methylated DNA and proteins that constitute chroma-
minority. Methylation of histones was shown to be essential for
triggering DNA methylation in filamentous fungi, and it was
suggested that propagation of DNA methylation patterns is
dependent on feed-back loops between modification of chroma-
tin proteins and DNA (35). Our result showing periodic oscil-
lolation of DNA methylation within the nucleosomal structure is
consistent with this view and further suggests a positive role
for methylation in determining the chromatin infrastructure.
Our findings also substantiate occurrence of demethylation upon environmental stress.

During differentiation of mammalian cells, genome-wide loss
of DNA methylation occurs, for which active or passive mecha-
isms have been proposed (11, 36). In active demethylation,
mC is enzymatically removed from DNA, and in passive dem-
ethylation, newly replicated DNA is not methylated (11). Our
results with maize indicate that genome-wide demethylation occurs, possibly through active demethylation, because chilled
tissues immediately ceased DNA replication (28). A rapid and
active decrease in global DNA methylation was also observed
during seed germination of Silene latifolia (37). Active
demethylation is catalyzed by 5-methylcytosine DNA glycosidase
(11, 36), although such activity has not yet been found in plants. A specific feature of our findings was that demethylation
predominantly occurred at the nucleosome core regions as
revealed by direct methylation mapping. A question arises,
than, as to how such a differential demethylation pattern is
formed. Currently we have no explanation for this, but some clues for speculation are available. For example, nucleosome
cores are proposed to be located outside the 30-nm fiber, which is
composed of a unit of six nucleosomes. Linker regions are
connected by histone H1 inside the fiber. This structure makes
5-methylcytosine DNA glycosylase more accessible to the core
than to the linker. Alternatively, 5-methylcytosine DNA glyco-
sylase may be selectively recruited to the core by specific pro-
tein(s) such as, for example, 5-methylcytosine binding proteins.
Another distinct finding of the direct methylation mapping is
ubiquitous demethylation at CpG, CpNpG and other sites, sug-
showed that this can indeed occur. However, maize roots are
transmittable, but evidence of methylation patterns changing
type, both of which are heritable (20).
changed expression of some genes, resulting in a new pheno-
esis those that are acquired are usually completely erased and
altered phenotypes, including dwarfism. The acquired traits
chemical that powerfully induces demethylation of DNA
between somatic and germ cells is less obvious in comparison
primary activation/inactivation of other genes (41). However,
development the methylation/demethylation system to simulta-
plants may express as many as several hundred genes upon
matin structure and thus influence gene expression. Increase in
methylation in cold-chased seedlings.
Type alteration of DNA methylation can induce
and demethylation patterns were inherited for up to at least six
genomes.2 Thus alteration of DNA methylation can induce
chemicals, making possible transmission of acquired DNA
reason why such demethylation occurs only in root tissues
is not clear. One explanation might be that above-ground parts
is not clear. One explanation might be that above-ground parts
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published observation.
demethylation was observed in stem mesocotyl tissues, which
contain cells with the potentiality to develop into germ cells,
suggests that these latter are well protected against environ-
mental stresses in terms of DNA methylation. However, if a
DNA methylation pattern did change, it could result in herita-
ble epimutations. If such mutations were advantageous for
survival, they might persist for generations. An example is the
flower morphology change in Lianaria vulgaris, considered to be
the result of hypermethylation of the Lyc gene which occurred
250 years ago (42). Also, in plants, vegetative reproduction is
not rare as seen with tuber propagation, making it possible
to directly transmit altered methylation patterns to the next
generation. We therefore speculate that Lamarkian inheritance
does exist, being mediated through DNA methylation.

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