Transcriptional regulation and DNA methylation of nuclear genes for photosynthesis in nongreen plant cells*

(*Acer pseudoplatanus*/suspension-cultured cells/nuclear gene expression/HeLa cell lysate/*in vitro* transcription)

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ABSTRACT The transcripts of nuclear genes for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*), chlorophyll a/b-binding protein (*cab*), and extrinsic 33-kDa protein involved in photosystem II water oxidation (*wxa*) were not detectable in the white wild cultured cells of sycamore (*Acer pseudoplatanus*), in contrast to their high levels in the sibling green mutant cells and the constitutive expression of actin genes (*act*) in both cell types. We have examined the template activities of nuclear DNAs using the HeLa cell *in vitro* transcription system. All of the three photosynthesis genes from the green cell line and *act* from both cell types were well transcribed *in vitro*, but these photosynthesis genes from the white cell line were not, indicating that the transcriptional regulation is ascribable to DNA templates. Digestion of nuclear DNA with methyl-sensitive and -insensitive isoschizomeric endonucleases and the subsequent Southern hybridization showed that each gene has the identical recognition sites of restriction enzymes in the green and white cell lines, but some of the sites were methylated only in the photosynthesis genes in the white cells. There was observed a clear inverse relationship between the level of expressed transcripts and the extent of DNA methylation. Thus, it is inferred that the selective methylation of DNA is a likely mechanism for suppressing transcription of nuclear genes for photosynthesis in the nonphotosynthetic plant cells.

Differentiation of various types of plastids such as the developmental formation of chloroplasts in greening seedlings and the transitional formation of chromoplasts from chloroplasts during tomato fruit ripening contains complicated processes. There exists interaction of nuclear and plastid genes, and one or more regulatory mechanisms operating therein are not thoroughly understood, although it is well recognized that the coordinated expression of these two genome types is required in the overall process.

The transcriptional, posttranscriptional (1, 2), and translational (3, 4) controls as well as posttranslational regulation (5) have been postulated as mechanisms governing the expression of photosynthesis genes during plastid differentiation, and along this line of research we have selected liquid-cultured cells of sycamore, plane maple (*Acer pseudoplatanus*) as an experimental system. The white wild cell line of sycamore grows heterotrophically, whereas the sibling green mutant cell line originally isolated from the former by the mutagen treatment (6) can be cultured under complete autotrophic condition and absolutely requires light illumination (7). We have previously reported that some polypeptides such as ribulose-1,5-bisphosphate carboxylase/oxygenase [RbuP₂Case; 3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] cannot be detected in the white wild cells (7), and it was subsequently found that the chloroplastic gene

(*rbcL*) encoding the large subunit of RbuP₂Case is little expressed in the amyloplasts (8). However, the amyloplasts were competent to import and proteolytically process the precursors of polypeptides synthesized in the cytoplasm such as the small subunit of RbuP₂Case encoded by the nuclear gene *rbcS* (9). Thus, it is imperative for us to examine whether these genes are transcribed in the white wild cells; if not, a pertinent question to be answered is what regulatory mechanisms may govern the overall processes.

In the present investigation, we have attempted to study the rate-limiting step involved and have examined the transcriptional template activities of nuclear genes from both the white wild and green mutant cell lines of sycamore. We report here experimental results supporting the view that the selective methylation of nuclear DNA plays a causative role in the transcriptional suppression of nuclear genes for photosynthesis in the nonphotosynthetic plant cells.

MATERIALS AND METHODS

Cell Cultures. The white wild cell line of sycamore was grown in a liquid medium by supplementation with sucrose and 2,4-dichlorophenoxyacetic acid (2,4-D) as described by Bligny (10). The green mutant cell line of sycamore originally isolated by Lescure (6) was grown without 2,4-D under continuous illumination with fluorescent light (2,000 lux) at 25°C. At the exponential stage of growth, the cells were harvested and processed for the isolation of nuclear DNA and total cellular RNA.

Nuclear DNA and Total Cellular RNA Preparation. To purify nuclear DNA, nuclei were isolated from protoplasts of the two cell lines, and the DNA was purified by CsCl density gradient centrifugation as reported (11). Each batch of purified nuclear DNA was ascertained to be free from contamination of plastid DNA by Southern blotting with a labeled *rbcL* gene probe. Total cellular RNA from both cell lines was isolated by the reported method (7) in the presence of an RNase inhibitor from human placenta (Takara Shuzo, Kyoto, Japan).

Characterization of RNA and DNA. Purified cellular RNA was denatured by glyoxal and electrophoresed in an agarose gel. The digestion of DNA with several restriction endonucleases and subsequent agarose gel electrophoresis were performed by the conventional techniques as described (8). Transfer of DNA and RNA to Zeta-Probe (Bio-Rad) membranes and subsequent hybridization were as described in Bio-Rad's instruction manual(s). The radioactive bands on the membranes were detected by Fuji x-ray film RXO-G with Fuji intensifying screen GRENE-X and exposure at -80°C.

Abbreviation: RbuP₂Case, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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Plasmids bearing pea leaf cDNA clones for *rbcS* (pSS15) and *cab*, which encodes the chlorophyll a/b-binding protein (pAB96) (12, 13), and for *woxA* (see ref. 14 for gene designation), which encodes the extrinsic 33-kDa protein involved in the photosystem II water oxidation (pWOX338) (15), were used throughout this investigation. The plasmid containing 18S rRNA-encoding DNA from the fungus *Alternaria alternata* (pABM4; unpublished data), specifically hybridizable with higher plant 18S ribosomal DNAs, was also used. DNA probes were labeled with [α - 32 P]dCTP by using the Klenow fragment of *Escherichia coli* DNA polymerase I and primers in the oligolabeling kit of Pharmacia. Oligonucleotides complementary to conserved sequences of dicotyledon actin gene (*act*) corresponding to near N-terminal regions (16–18), 5'-ACCATTC(AT)GTTCCATTGTC(AG)CAAACAAG-3' in which parentheses show mixtures of two bases in these positions, was synthesized by a DNA synthesizer (Applied Biosystems model 381A); the oligonucleotide was purified by OPC Cartridge (Applied Biosystems) and was labeled at the 3' end by T4 polynucleotide kinase with [γ - 32 P]ATP (222 TBq/mmol).

In Vitro Transcription by HeLa Cell Lysate. The HeLa cell lysate was supplied by BRL (19). The *in vitro* transcription assay of nuclear DNA from both cell lines was carried out with the HeLa cell lysate at 30°C for 1 hr following the published procedure (20) except that RNase inhibitor from human placenta was added. The resultant radioactive transcripts purified as described (8) were assayed to determine the incorporation of [α - 32 P]UTP into RNA. Ten microliters of the extract after phenol/chloroform treatment was spotted on a Whatman GF/C glass-fiber disc, which was washed five times with cold 5% (wt/vol) trichloroacetic acid, twice with 95% ethanol, and finally dried.

Size Analysis of *in Vitro* Transcription Products. Nonlabeled transcripts were produced by the HeLa *in vitro* transcription system as described above, except for the use of nonradioactive UTP. To compare the size of transcripts, both *in vitro* transcripts and total cellular RNA were subjected to denaturation with glyoxal and subsequent electrophoresis in a 1.2% agarose gel, followed by RNA-DNA hybridization with labeled DNA probes as described (7). In the case of hybridization with oligonucleotide-end-labeled probes, the blots were hybridized as described in Bio-Rad's instruction manual for 24 hr at 56°C (21).

RESULTS

Absence of Transcripts from Nuclear Genes for Photosynthesis in White Wild Cell Line. We first examined the expression of nuclear genes for photosynthesis in both white wild and green mutant cells of sycamore. The levels of transcripts for *rbcS*, *cab*, *woxA*, and 18S rRNA genes in total cellular RNA preparations were determined by RNA-DNA hybridization analysis. The green mutant (M lanes 4, 6, 8, and 10) displayed clear positive signals with the DNA probes tested (Fig. 1). In contrast, transcripts for *rbcS*, *cab*, and *woxA* were not detectable in the white wild cells (W lanes 5, 7, and 9). The hybridization with 18S rRNA gene probe showed that 18S rRNA was present in both the green mutant (lane 10) and white wild (lane 11) cells.

Template Activity of Nuclear DNA by HeLa *in Vitro* Transcription System. To examine mechanisms of transcriptional regulation, we determined the template activities of nuclear DNAs from both cell lines using a heterologous *in vitro* transcription system by HeLa cell lysate. In the assay, *Pst* I-cleaved simian virus 40 was used as a positive control as described by Handa *et al.* (20). The reaction was carried out with [α - 32 P]UTP as substrate, and the resultant radioactive products were subjected to analysis for 32 P incorporation into the trichloroacetic acid-insoluble fraction and subsequent

FIG. 1. RNA-DNA hybridization analysis of total cellular RNAs isolated from green mutant (lanes M) and white wild (lanes W) cell lines of sycamore with cDNA probes. The agarose gel (1.2%) electrophoretic patterns of purified total cellular RNA (20 μ g each) from two cell lines are represented in lanes 2 and 3. (a–d) Autoradiograms of the RNA-DNA hybridization, with nuclear cDNA probes specified at the bottom of the lanes. The sizes of RNA size markers from HeLa cells (28S, 18S rRNA) and *E. coli* (23S, 16S rRNA) are indicated at the left (lane 1).

hybridization with the membrane-blotted plasmids containing DNA probes.

The addition of α -amanitin (0.5 μ g/ml), an inhibitor of RNA polymerase II (19), drastically inhibited the 32 P incorporation (99%), showing that RNA polymerase II entails the transcriptional activity (Table 1). The degree of incorporation was indistinguishable between nuclear DNAs derived from the two sycamore cell lines. The cell line-specific factor(s) inhibiting transcription appeared to be absent in DNA preparations, since the total incorporation of 32 P was barely altered when the mixture of the two preparations was used as template in the *in vitro* transcription system.

The labeled transcripts for *rbcS*, *cab*, and *woxA* were obtained from the *in vitro* transcription system when the nuclear DNA from green mutant was used as the template. In contrast, none of these products were detectable with the nuclear DNA of white wild cells (data not shown). It should be noted that the 18S rDNA was shown not to serve as a template in the *in vitro* transcription system, consistent with the previous report showing that rDNA can be transcribable only by RNA polymerase I (22).

Size Analysis of Transcripts Synthesized *in Vitro*. The sizes of the transcripts produced in the *in vitro* transcription

Table 1. Template activity of nuclear DNAs obtained from green mutant and white wild cultured cells of sycamore in the HeLa *in vitro* transcription system

DNA templates*	32 P incorporation,† cpm	
	Without α -amanitin	With α -amanitin (0.5 μ g/ml)
No DNA	4.71×10^4	4.84×10^4
SV40 DNA‡	8.28×10^6	1.24×10^5
Green mutant nuclear DNA	2.67×10^6	2.40×10^4
White wild nuclear DNA	2.59×10^6	2.85×10^4
Green mutant DNA + white wild nuclear DNA	2.69×10^6	2.62×10^4

*Purified DNA (1 μ g) was used in each assay.

†Total incorporation of 32 P into trichloroacetic acid-insoluble fractions following the *in vitro* transcription assay was as described.

‡*Pst* I-cleaved SV40 DNA was used as a quality control.

FIG. 2. Size analysis of *in vitro* transcripts and total cellular RNAs for *rbcS* (a), *cab* (b), *woxA* (c), and *act* (d). RNA-DNA hybridization of cellular RNAs (0.5 μ g) from green mutant (M lanes 1) and white wild (W lanes 2) cells and nonradioactive *in vitro* transcripts generated by using 4 μ g each of nuclear DNAs from green mutant (M lanes 3) and white wild (W lanes 4) cells as templates in the HeLa *in vitro* transcription system. Autoradiograms of hybridization with labeled DNA probes are shown. Migrating positions of RNA size markers, 18S rRNA from HeLa cells and 16S rRNA from *E. coli*, are shown at the left. The approximate sizes of transcripts are indicated at the right.

system were compared with those in the total cellular RNA as shown in Fig. 2. The approximate sizes of transcripts for *rbcS* (Fig. 2a), *cab* (Fig. 2b), *woxA* (Fig. 2c), and *act* (Fig. 2d) were 1.0, 1.2, 1.2, and 1.7 kilobases (kb), respectively. The sizes of transcripts for *rbcS*, *cab*, and *act* were nearly identical to those reported in several other plant species (23, 24). Furthermore, they are identical between the total cellular RNA (lanes 1 and 2) and the *in vitro* transcription products using the nuclear DNA derived from both cell lines (lanes 3 and 4). The transcripts of *rbcS*, *cab*, and *woxA* were not detectable either in the cellular RNA or the *in vitro* tran-

scripts of the white wild cells (lanes 2 and 4). However, the presence of transcripts of *act* indicated that the housekeeping genes in white wild nuclear DNA are actively transcribed (Fig. 2d, lanes 2 and 4).

Identical Restriction Sites in Nuclear DNAs from Two Cell Lines and Methylated Sequences in Lowly Expressed Genes. To examine the identity of nucleotide sequences between the nuclear DNAs of both cell lines and the distribution of methylated bases, we performed the Southern blot analysis of DNA fragments produced by three pairs of methyl-sensitive and -insensitive isoschizomeric restriction enzymes—i.e., *Bst*NI/*Eco*RII, *Msp* I/*Hpa* II, and *Sau*3AI/*Mbo* I. It is known that *Bst*NI can cleave the 5'-CCA/TGG-3' sequences when the internal cytosine residue is 5-methylcytosine, while *Eco*RII cannot. *Msp* I and *Hpa* II can cleave the 5'-CCGG-3' sequences containing a methylated cytosine residue at different positions. In another pair of isoschizomers, *Sau*3AI can cleave the 5'-GATC-3' sequences containing *N*⁶-methyladenine, but *Mbo* I cannot (25).

The hybridization data presented in Fig. 3 clearly show multiple signals with respect to each individual cDNA probe tested, indicating the presence of multigene families as reported (26). The digestion of DNAs with the methyl-insensitive enzymes exhibited the completely identical profiles between the two cell lines. Sizes of DNA fragments produced by the pairs of methyl-sensitive and -insensitive isoschizomers hybridizable with each specific gene probe were identical in the green mutant, showing the absence of methylated bases in the recognition sequence. In contrast, the profiles were different from that of the nuclear DNA of white wild cell line, showing the presence of 5-methylcytosine or *N*⁶-methyladenine in the recognition sites in *rbcS*, *cab*, and *woxA* regions (Fig. 3) but not in the *act* region (data not shown). It must be emphasized that the unique fragments produced by the methyl-sensitive enzymes persisted regardless of the extended time of digestion and the amount of enzymes; absence of any inhibitors for the restriction enzymes was ensured by the codigestion of plasmid pBR322 and the subsequent hybridization experiments (data not shown).

Methylated Base Compositions of Nuclear DNA. The purified nuclear DNAs from both cell lines were completely hydrolyzed and subjected to base composition analysis by

FIG. 3. Methylation of nuclear DNA regions containing photosynthesis genes. Nuclear DNAs (1 μ g) from green mutant (lanes M) and white wild (lanes W) cell lines were digested with three pairs of isoschizomeric endonucleases: *Bst*NI (B lanes 1 and 3)/*Eco*RII (E lanes 2 and 4); *Msp* I (Ms lanes 5 and 7)/*Hpa* II (H lanes 6 and 8); and *Sau*3AI (S lanes 9 and 11)/*Mbo* I (Mb lanes 10 and 12). DNA fragments electrophoresed in agarose gel (0.7%) were transferred to Zeta-Probe membranes and subjected to the Southern hybridization with the DNA probes as shown. Different specific cleavage sites observed only in the white wild nuclear DNA are marked by white circles. The sizes of the DNA fragments are shown at the left in kilobase pairs.

reverse-phase HPLC (8). The mol % of bases were calculated by standardization of each peak area with respect to the standard bases (Table 2). A variety of methylated bases were detected, and the mol % of methylated bases were indistinguishable between both cell lines. The base compositions of salmon sperm DNA in the analysis always agree with the published data, and the G+C content of sycamore nuclear DNA was found to be in a range of ordinary values in higher plants (27). Among several methylated bases, 5-methylcytosine was shown to be most abundant in the DNAs from both cell lines, accounting for 25% of the total cytosine residues.

DISCUSSION

It is absolutely necessary to use the nuclear *in vitro* transcription system to examine whether or not a regulatory factor is associated with the DNA template. A reliable *in vitro* transcription system of plant origin is not readily available, although wheat germ is reported to be a useful material (28, 29). In the present study, we have used a heterologous *in vitro* transcription system containing HeLa cell lysate. Transcription of the maize *zein* gene by the HeLa cell lysate has been reported (30). As presented in Fig. 2, the lysate was able to transcribe effectively four kinds of plant genes. Interestingly, termination, polyadenylation, and correct splicing appeared to proceed as ascertained from the determined transcript sizes. Although it has been reported that the HeLa cell lysate entails the activities of termination (31), polyadenylation (32), and splicing (33) of animal or plant genes, these reactions had not been examined in a single reaction system. Thus, this report shows the most satisfactory use of the HeLa cell lysate in plant gene transcription and subsequent RNA processing.

The postreplicative methylation of DNA has been postulated to entail many important biological functions including its regulatory role in the gene expression in both prokaryotic and eukaryotic cells (34). Although it has been proposed that the transcriptional activities of certain vertebrate genes are closely correlated with the reduced levels of methylation (34–36), detailed molecular mechanisms have not yet been fully understood (37). Various patterns of methylation of nuclear DNA have been reported in plant cells (38), and it has indeed been known for a long time that plant DNAs contain abundant 5-methylcytosine compared to the mammalian

DNAs (27). However, so far there is little information available concerning possible functions of this modified base. Recently, it has been reported that the methylation of genes for tissue-specific storage proteins in maize correlates with their reduced transcriptional activities (39). Studies on the T-DNA of crown gall tumors (40) and the ribosomal genes (41) also showed that there is an inverse relationship between the magnitude of methylation and the transcriptional activity. We have reported that DNA methylation serves as a mechanism for suppressing the transcription of photosynthetic genes in nongreen plastids—i.e., amyloplasts in white wild cultured cells of sycamore (7, 8) and chromoplasts in ripened red tomato fruits (42). Furthermore, a role of DNA methylation has been found in the differential expression of *C₄* photosynthesis genes in mesophyll and bundle sheath cells of greening maize leaves (43).

Bearing in mind the limitations of the heterologous *in vitro* transcription assay system used in the present investigation, we hypothesize from our experimental results that the direct interaction between the region of nuclear DNA containing the selectively methylated bases and either RNA polymerase or common transcription-regulating factor(s) is involved in the specific transcriptional suppression of photosynthesis genes in the white wild sycamore cells. To our knowledge, there has not been reported any evidence showing the direct interaction between methylated plant DNA and RNA polymerase II, whereas there are some contradictory observations in animal cells. It has been reported that methylated simian virus 40 and adenovirus 2 DNA can be normally transcribed by HeLa cell lysate (44, 45), whereas the methylated DNA microinjected into the frog oocytes was poorly transcribed (44, 46). The interaction between DNA and nuclear proteins but not RNA polymerase II is postulated to be affected by DNA methylation in vertebrates (47). Attempts to determine the specific sites of methylated sequences around the promoter region of nuclear DNA interacting with RNA polymerase II or other factors in the white wild cells of sycamore stand a good chance of clarifying the complicated regulatory process of gene expression in plant cells in general at the molecular level.

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Table 2. Base compositions of nuclear DNAs obtained from green mutant and white wild cultured cells of sycamore

Base	Base composition, mol %		
	Salmon sperm	Green mutant cells	White wild cells
Cytosine	18.01	13.29	13.16
3-Methylcytosine	0.56	1.42	1.44
5-Methylcytosine	1.68	5.65	5.79
Guanine	21.59	18.26	18.17
1-Methylguanine	ND	ND	ND
3-Methylguanine	ND	ND	ND
7-Methylguanine	0.11	0.24	0.27
<i>N</i> ² -Methylguanine	ND	1.01	1.05
Adenine	28.74	29.68	29.11
1-Methyladenine	ND	ND	ND
<i>N</i> ⁶ -Methyladenine	ND	ND	0.49
Thymine	29.31	30.45	30.52
Total	100.00	100.00	100.00

The mol % values were calculated by standardization of each elution peak area (*A*₂₅₄) relative to that of a standard mixture of four major bases and eight methylated bases (see figure 3 in ref. 8). Acid hydrolysates of DNA (5 µg) were subjected to analysis, and each value represents an average of three separate determinations. ND, not detectable (<0.02%).

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