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Communication

DNA Methylation Occurred around Lowly Expressed Genes of Plastid DNA during Tomato Fruit Development[1]

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ABSTRACT

We have analyzed DNA methylation of plastid DNA from fully ripened red fruits, green mature fruits, and green leaves of tomato (*Lycopersicon esculentum* var. Firstmore). Essentially identical restriction profiles were obtained between chromoplast and chloroplast DNAs by *Eco*RI digestion. *Bst*NI/*Eco*RII and *Hpa*II/*Msp*I are pairs of isoschizomers that can discriminate between methylated and unmethylated DNAs. These endonucleases produced different restriction patterns of plastid DNAs from tomato fruits compared to tomato leaves. Moreover, we have found from Southern blots that methylation was not detected in DNA fragments containing certain genes that are actively expressed in chromoplasts, whereas DNA fragments bearing genes that are barely transcribed in chromoplasts are methylated.

Regulatory mechanism(s) governing expression of plastid genes during differentiation has received a great deal of attention (5, 20). The transformation of etioplasts (proplastids) to chloroplasts upon illumination of dark-grown seedlings and that of chloroplasts to chromoplasts in fruit tissues, such as tomato and *Capsicum* spp., are well characterized examples of organelle differentiation (1, 3, 6, 7, 19, 20, 26). Several investigators have reported that the restriction profiles of DNA isolated from the chromoplasts are identical with those of chloroplast DNA (6, 8, 9, 24). Piechulla *et al.* (15, 16) have demonstrated that the levels of most of transcripts for both chloroplast- and nucleus-encoded photosynthesis-specific genes in chromoplasts are lowered in the development of fruits.

We have found that the transcript levels in amyloplasts in a heterotrophic cell line of sycamore (*Acer pseudoplatanus* L.) are markedly low in comparison with those in chloroplasts in a photoheterotrophic green mutant cell line (11–14). Although the restriction patterns of the two classes of plastid DNAs with *Eco*RI were identical, we found evidence for methylation in DNA regions containing *rbcL* (large subunit of RuBisCO[3]), *atpA* (α subunit of CF_1), *atpB* (β-subunit of CF_1), *atpE* (ϵ-subunit of CF_1), *psaA* (apoprotein of P700), and *rps4* (ribosomal protein S4) in amyloplast DNA but not in chloroplast DNA (13). Furthermore, those genes in amyloplasts are inactive as DNA template in *in vitro* transcription, but those in chloroplasts are active (13). Interestingly, we have been unable to detect methylation in regions of amyloplast DNA containing 16S rDNA and *psbA* (PG32), the transcripts of which are not low *in vivo*, and indeed these two genes are actively transcribed in the *in vitro* transcription.

These observations strongly suggest that methylation of DNA may constitute a regulatory mechanism for inhibiting the expression of amyloplast DNA in sycamore cells. Considering that the relationship between amyloplasts and chloroplasts in sycamore is analogous to that of etioplasts and chloroplasts in the greening plant tissues, it must be clearly recognized that the sycamore amyloplasts are unable to transform to chloroplasts upon illumination of the cultured cells. It appears to be important to test whether DNA methylation might be a general mechanism in controlling gene expression. We report here DNA methylation in the chromoplast DNA during tomato fruit ripening as one of the possible causes of the regulation of gene expression.

MATERIALS AND METHODS

Plant Materials and Chromoplast Isolation. Mature green and fully ripened red fruits of tomato (*Lycopersicon esculentum* var. Firstmore) as well as green leaves harvested at the Nagoya City Agriculture Center were used throughout this investigation. The isolation of chloroplasts and chromoplasts was carried out employing the discontinuous sucrose gradient centrifugation technique, based on the method described by Bathgate *et al.* (2), followed by centrifugation in Percoll gradient (9). As a control, chloroplasts were isolated from leaf tissues following the method of Takabe *et al.* (22).

DNA Isolation and Endonuclease Digestion. Chloroplast and chromoplast DNAs were isolated by centrifugation in CsCl gradients and purified to homogeneity by three repeated centrifugations.

Chloroplast or chromoplast DNA (1 μg each) was digested by various restriction endonuclease (10 units each) for 2 h as specified by the manufacturers. DNA fragments were electrophoretically separated on 0.7% agarose gels in the presence of ethidium bromide. Pairs of isoschizomers, which respond differentially to methylation: *Eco*RII and *Bst*NI, *Msp*I and *Hpa*II, *Mbo*I, and *Sau*3AI, were used to detect methylated residues of DNA derived from the two types of plastids (see Fig. 1).

Southern Hybridization Experiments. In order to identify methylated genes, digested DNA fragments were transferred to GeneScreen (New England Nuclear) and subjected to the South-

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[3] Abbreviations: RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase; CF_1, coupling factor of photosynthesis; PG32, 32-kD Q_B protein.

ern hybridization (21). Cloned fragments of maize chloroplast genes were used throughout this investigation by permission of Dr. L. Bogorad of Harvard University as follows (10): RuBisCO large subunit (*rbcL*, pZmc461), PG32 (*psbA*, pZmc427), α-subunit of CF_1 (*atpA*, 0.6-kbp *Hin*dIII fragment of pZmc527), β- and ϵ-subunits of CF_1 (*atpB,E*, pZR4876), apoprotein of P700 (*psaA*, pZmc556), ribosomal protein S4 (*rps*4, pZmc747), and 16S rDNA (pZmc532). No hybridization of the plastid with plasmid vectors was observed. Plasmids were labeled with [α-^{32}P]dCTP *in vitro* using the Klenow fragment of *Escherichia coli* DNA polymerase I and primers provided in the Oligolabeling Kit (Pharmacia). Radioactive bands on the GeneScreen were detected upon exposure to x-ray film.

RESULTS AND DISCUSSION

We set out to test the possibility that selective methylation of plastid DNA may occur during the differentiation of chromoplasts. We first compared restriction patterns of plastid DNAs derived from three tissues of tomato plants: leaves, green fruits, and red fruits.

As shown in lanes 1, 2, and 3 of Figure 1B, the patterns of the restriction fragments produced by digestion of the three plastid DNAs with *Eco*RI were indistinguishable. Identical restriction patterns of tomato chloroplast and chromoplast DNAs with *Eco*RI have been reported previously (8, 9). There are, however, differences between the *Eco*RI patterns reported here and those of others because of the use of different cultivars. In view of the fact that the estimation of the size of our plastid DNA from the *Eco*RI digestion patterns is untenable presumably because of the presence of comigrating multiple fragments, we have digested the leaf chloroplast DNA by *Hpa*I, *Sal*I and *Eco*RI (Fig. 1A). The estimated size of the sample by *Hpa*I or *Sal*I digestion, 141.6 or 141.1 kbp, was found to be in a range of the ordinary size of chloroplast DNA.

We then examined restriction patterns of plastid DNAs using isoschizomers which differ in their sensitivities to methylation: (a) *Bst*NI and *Eco*RII, (b) *Msp*I and *Hpa*II, and (c, *Mbc*I and *Sau*3AI. *Eco*RII cannot cleave the 5′-CCA/TGG-3′ sequence when the internal cytosine residue is methylated, whereas *Bst*NI can. Chloroplast DNA from green leaf tissues gave the identical digestion patterns with the two enzymes (lanes 4 and 5). In contrast, chromoplast DNAs from red or green fruits were completely digested by *Bst*NI, whereas only partially digested by *Eco*RII (see white open circles in lanes 6 through 9). *Msp*I and *Hpa*II cleave 5′-CCGG-3′ sequences containing a methylated cytosine residue at different position. The several kinds of plastid DNAs behaved somewhat differently toward this pair. Identical restriction patterns were obtained with leaf chloroplast DNA (lanes 10 and 11) and fruit chloroplast DNA (lanes 12 and 13), but distinct patterns were obtained with fruit chromoplast DNA

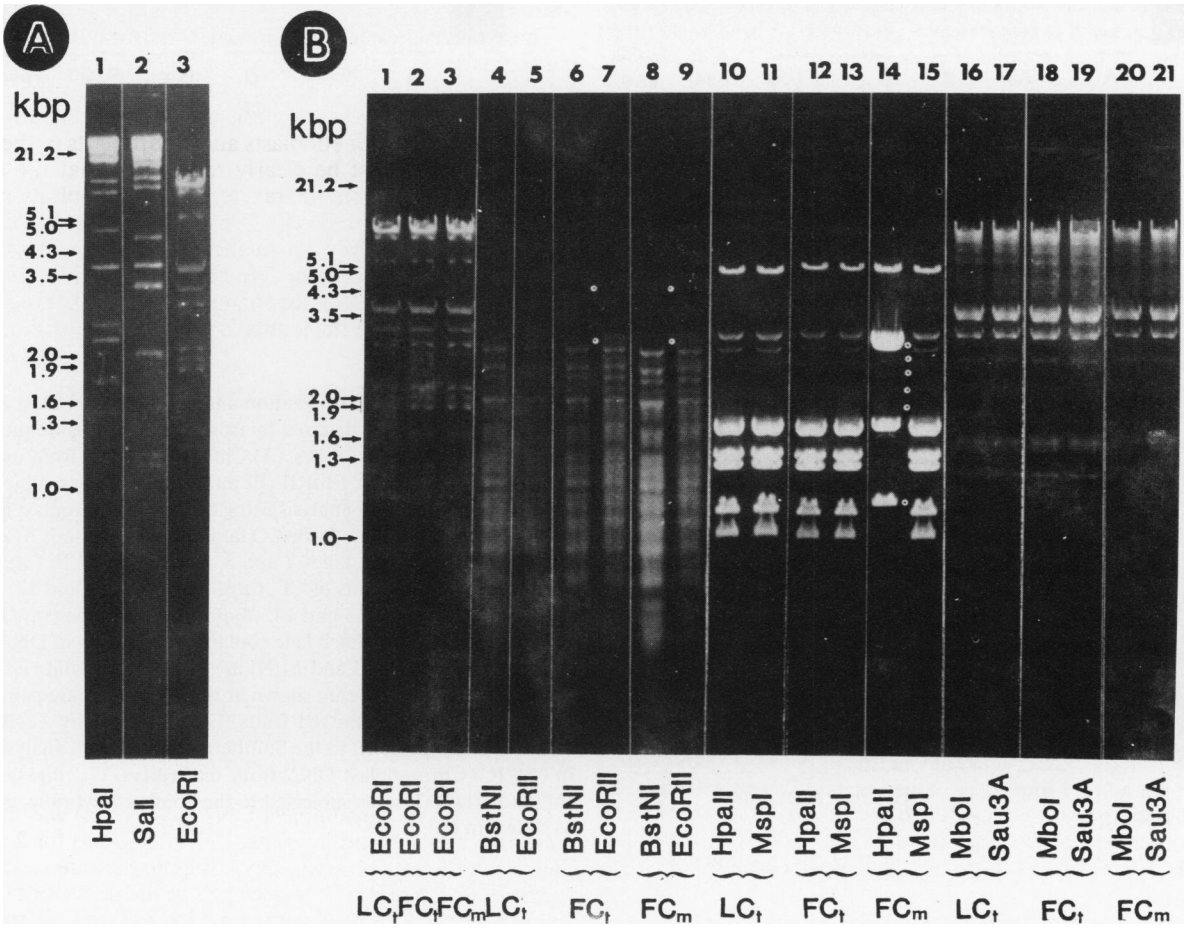

FIG. 1. Methylation analysis of chromoplast and chloroplast DNAs of tomato. (A) Electrophoretic patterns of chloroplast DNA from green leaves on 0.7% agarose gel. Chloroplast DNA (1 μg each) digested by HpaI (lane 1), *Sal*I (lane 2), and *Eco*RI (lane 3). (B) LC_t, chloroplast DNA from green leaves; FC_t, chloroplast DNA from mature green fruits; FC_m, chromoplast DNA from red fruits. DNA samples (1 μg each) were digested with various endonucleases (10 units each, 2 h, 37°C) as indicated and subjected to 0.7% agarose gel electrophoresis. *Eco*RI and three pairs of isoschizomers of endonuclease (*Bst*NI/*Eco*RII; *Hpa*II/*Msp*I; *Mbo*I/*Sau*3AI) were used to identify sites of methylation in the DNAs. Different cleavage sites are indicated by white open circles. DNA sizes are shown at the left in kilobase pairs (kbp).

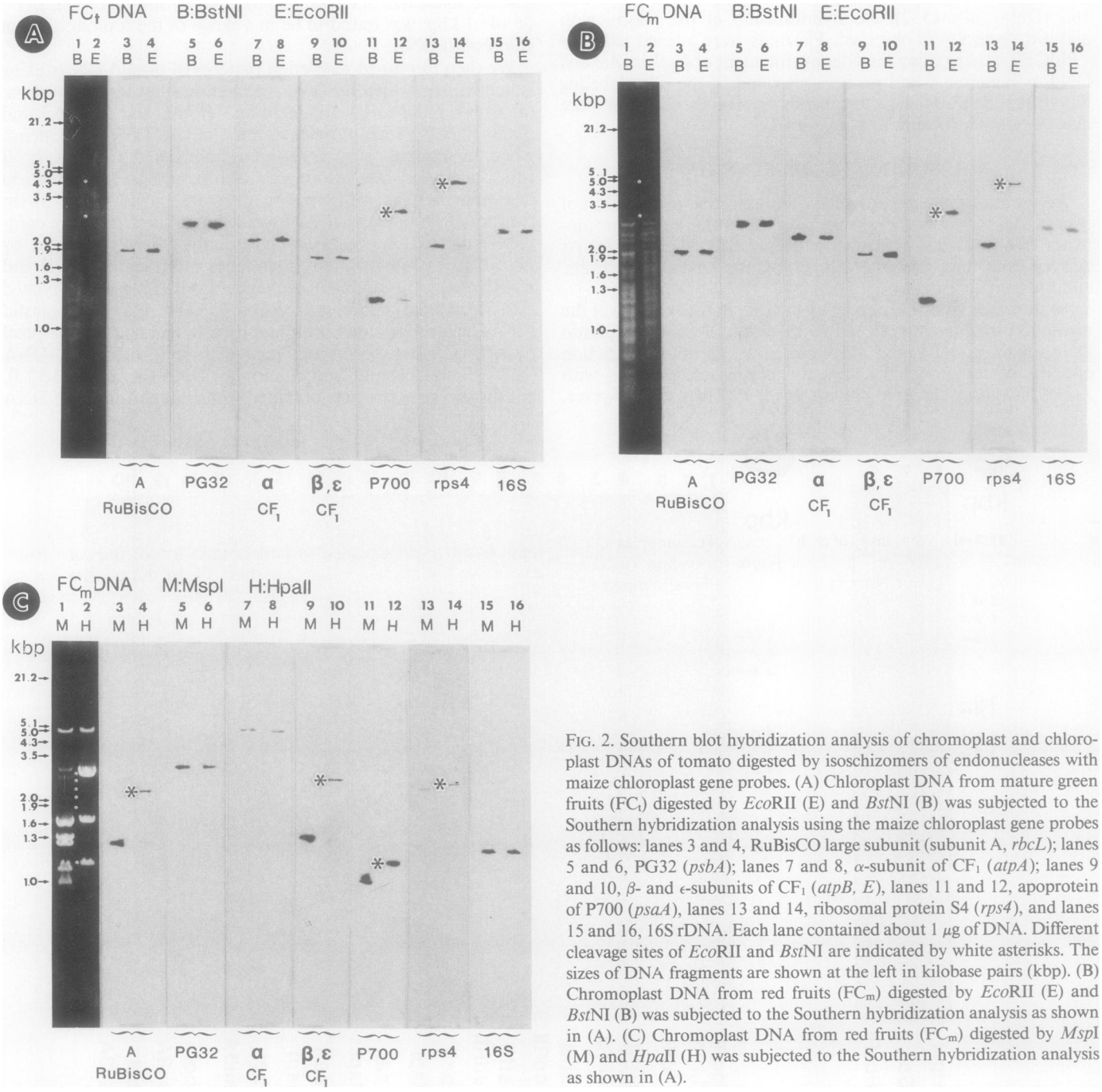

FIG. 2. Southern blot hybridization analysis of chromoplast and chloroplast DNAs of tomato digested by isoschizomers of endonucleases with maize chloroplast gene probes. (A) Chloroplast DNA from mature green fruits (FC_t) digested by *Eco*RII (E) and *Bst*NI (B) was subjected to the Southern hybridization analysis using the maize chloroplast gene probes as follows: lanes 3 and 4, RuBisCO large subunit (subunit A, *rbcL*); lanes 5 and 6, PG32 (*psbA*); lanes 7 and 8, α-subunit of CF_1 (*atpA*); lanes 9 and 10, β- and ϵ-subunits of CF_1 (*atpB, E*), lanes 11 and 12, apoprotein of P700 (*psaA*), lanes 13 and 14, ribosomal protein S4 (*rps4*), and lanes 15 and 16, 16S rDNA. Each lane contained about 1 μg of DNA. Different cleavage sites of *Eco*RII and *Bst*NI are indicated by white asterisks. The sizes of DNA fragments are shown at the left in kilobase pairs (kbp). (B) Chromoplast DNA from red fruits (FC_m) digested by *Eco*RII (E) and *Bst*NI (B) was subjected to the Southern hybridization analysis as shown in (A). (C) Chromoplast DNA from red fruits (FC_m) digested by *Msp*I (M) and *Hpa*II (H) was subjected to the Southern hybridization analysis as shown in (A).

Table I. *Quantitative Analysis of DNA Fragments Generated by Isoschizomers*

Fragment[a]	*Bst*NI		*Eco*RII		b − d[e]
	Molar ratio[b]	Gene[c]	Molar ratio[d]	Gene[c]	
kbp					
4.3			1	*rps4*	−1
2.9			2	*psaA*	−2
2.8	2	*psbA*	2	*psbA*	0
2.7	2	*psbA*	2	*psbA*	0
	*Msp*I		*Hpa*II		
5.1	2	*atpA*	2	*atpA*	0
3.1	2	*psbA*	2	*psbA*	0
2.8	1		6<	*atpB, E*	−5>[f]
2.5			1	*rps4*	−1[g]
2.3	1	*rps4*	2	*rbcL*	−1[g]
2.1			1		−1[g]
1.9			2		−2[g]
1.8	1		8<		−7>[h]
1.7	12<				12<[h]
1.4	7<	*atpB, E*	2	(*atpE, B*)	5<[f]
1.3	6<	*rbcL*	3	(*rbcL*)	3<[f,g]
		16S		16S	
1.1	2		3		−1>[g]
1.04			8<	*psaA*	−8>[i]
1.03	8<				8<[g,i]
1.00	6<	*psaA*			6<[g,i]

[a] Sizes of chromoplast DNA fragments generated by the isoschizomers are given. For *Bst*NI and *Eco*RII, only largest four fragments are shown. [b] The relative intensity of each fragment of chromoplast DNA presented in Figure 1 was determined by densitometric tracing. The value was divided by the size of DNA fragment to get the relative number of fragments. The molar ratio, equivalent to the fragment number composing one chromoplast DNA, was then calculated from the relative number of fragments on the assumption that the total size of chromoplast DNA is 140 kbp. Since a linear relationship was obtained between the DNA content and the intensity of DNA fragment stained by ethidium bromide in a limited range of DNA content, strong bands were generally estimated lower, which are expressed with a symbol of inequality. [c] Gene locations on DNA fragments are based on results shown in Figure 2. [d] Same as "b." [e] Differences in the molar ratios, b and d, are given. Since *Eco*RII or *Hpa*II do not cut some methylated sequences, values below zero, and those larger than zero, mean numbers of fragments newly generated by *Eco*RII or *Hpa*II, and those of unmethylated sequences, are those missed by *Eco*RII or *Hpa*II, respectively. [f-i] DNA bands generated by *Msp*I or *Hpa*II are classified to four groups, namely "f" to "i." For example, in the case of "f," one 2.8-kbp fragment by *Hpa*II digestion is composed of 1.4- and 1.3-kbp fragments generated by *Msp*I. Although DNA fragments lower than 0.5 kbp were not detected, such short fragments are thought to exist explaining the whole results of this table.

(lanes 14 and 15). Sau3AI cleaves 5′-GATC-3′ sequences containing methylated adenine residue, but *Mbo*I does not. We obtained identical restriction patterns of all of the plastid DNAs with this pair of endonucleases (lanes 16 through 21).

In comparing the various restriction patterns, it appears that methylation of the 5′-CCA/TGG-3′ sequence occurs in plastid DNAs of both green and red fruits, but not in leaf chloroplasts. A sequence of 5′-CCGG-3′ appears to be methylated in plastid DNA of ripened red fruits, but not in chloroplast DNAs from leaves or green fruits. In none of the plastid DNAs is there methylation of the sequence 5′-GATC-3′. In order to determine how many sites are actually methylated, the restriction profiles given in Figure 1B were subjected to densitometric tracing. The relative intensities of each band determined were then subjected to calculation of the fragment numbers having specific size; results are summarized in Table I. It can be seen that, unusually, there are co-migrating bands, especially in the case of *Hpa*II and *Msp*I. Each faint band that is marked by a white open circle in Figure 1B is estimated to be composed of a singlet or doublet species.

In a second set of experiments, we looked for evidence that plastid genes undergo specific methylation in chromoplasts. In one experiment, DNAs from fruit chloroplasts and chromoplasts were digested with *Eco*RII and *Bst*NI, applied to electrophoresis, blotted and probed with plasmids containing fragments of *rbcL*, *psbA*, *atpA*, *atpB*, *atpE*, *psaA*, *rps4*, and 16S rDNA. The data of Figure 2, A and B, show that, except for *psaA* and *rps4*, each of the gene probes hybridized with the same sized fragments with the two endonucleases. In another experiment, Southern blots of fruit chromoplast DNA which had been digested with *Hpa*II and *Msp*I and probed with the same set of plasmids produced signals in fragments of different sizes for each of the genes except *psbA*, *atpA*, and 16S rDNA (Fig. 2C). These experiments provide the evidence for the uneven distribution of methylated bases in chromoplast DNA: 5′-CCA/TGG-3′ sequences appear to be methylated in or near *psaA* and *rps4*, and 5′-CCGG-3′ sequences appear to methylated around *rbcL*, *atpB*, *atpE*, *psaA*, and *rps4*. No methylation was detected in regions of *psbA* or 16S rDNA. The relationship between DNA fragments and their encoding genes is summarized Table I.

Results of Southern hybridization presented in Figure 2 show the presence of weak bands in addition to strong bands in the digestion by isoschizomers as marked by asterisks, because some methylated sequences at the same position cannot be hydrolyzable by the counterpart isoschizomers. Since the intensity of weak bands was estimated to be less than 5% of that of strong bands, we can calculate that less than 5% of the population of fruit chloroplast and chromoplast DNAs is not methylated in these specific regions.

The pattern of methylation of plastid genes in chromoplasts shown in Figure 2 correlates strikingly with measurements of the amounts of gene transcripts reported by Piechulla *et al.* (15, 16). They found that, in contrast to the high levels of transcripts for *psbA* and rDNA, transcripts for *psaA* and *rbcL* are little detectable in red fruits. We suggest that the observations of Piechulla *et al.* might be explained dealing with the inhibition of transcription of specific genes.

It has long been argued that methylation of DNA is a mechanism for the regulation of its biological functions (4, 23). In particular, there are indications that rates of transcription of some vertebrate (17) and plant (25) genes are closely related to reduced levels of methylation. Methylated DNA has not been reported in chloroplasts, except for the case of *Chlamydomonas reinhardtii*, in which maternal inheritance had been hypothesized (18). Together with our previous finding concerning the role of DNA methylation in the regulation of gene expression in sycamore amyloplasts (13), our present results provide additional evidence that methylation may regulate gene expression in another class of differentiated plastids.

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