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Chromoplast formation during tomato fruit ripening. No evidence for plastid DNA methylation

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Abstract

Ripening of tomato fruits involves differentiation of chloroplasts into non-photosynthetic chromoplasts. Plastid DNAs isolated either from green leaf chloroplasts or mature red fruit chromoplasts were compared by restriction endonuclease and DNA/DNA hybridization analyses. The same restriction and gene maps were obtained for both types of DNAs, illustrating the lack of major recombinational events during chromoplast formation. Several enzymes were used that discriminate the presence of methylated bases in their target sequences (*Pst* I, *Pvu* II, *Sal* I, *Mbo* I/*Sau* 3AI, *Msp* I/*Hpa* II, *Bst* NI/*Eco* RII). Plastid DNA fragments generated by these enzymes were hybridized against DNA probes encompassing about 85% of the tobacco chloroplast genome. These probes represented genes that follow very different expression behaviors in response to plastid development. Extensive restriction and hybridization analyses failed to reveal any difference between the chloroplast and chromoplast genomes, indicating that no developmentally related DNA methylation was detected by these methods. The results presented here do not support the hypothesis that selective DNA methylation of the chromoplast genome might play a major role in the transcriptional control of gene expression in these non-photosynthetic plastids.

Introduction

The process of fructification in higher plants is associated with dramatic structural and physiological changes, most remarkably the transition from chloroplasts to chromoplasts at the onset of fruit ripening [11]. Although it is not clear whether chromoplasts originate from the differentiation of a proplastid or the conversion of a fully developed chloroplast [5], chloroplasts from maturing tissues do undergo major changes, including breakdown of the thylakoid membranes and chlorophyll, mobilization of starch reserves and synthesis and accumulation of large amounts of carotenes [11].

Chromoplast development requires the coordinated expression of both nuclear and plastid (pt) genes to achieve stoichiometric accumulation of organelle-located peptides, and there are several indications that the biochemical changes observed during ripening are related to a decreased expression of ptDNA and of certain nuclear genes [9, 31, 32]. Piechulla and coworkers have shown that the steady-state levels of

chloroplast-specific transcripts and polypeptides (from both nuclear and plastid origin) are very low or undetectable in chromoplasts of mature tomato fruits [30–33]. Other nuclear-encoded mRNAs, however, are accumulated during ripening [11].

Inactivation of the plastid genes is apparently not due to major recombinational events at the level of ptDNA, since restriction enzyme patterns seem to be identical in chloroplast (cp) and chromoplast (cr) DNAs from tomato [14, 15], daffodil [13, 36], red pepper [10] and *Oenothera* [2]. However, recent reports indicate a higher level of methylation in tomato crDNA as compared to chloroplasts [16, 23], a phenomenon already reported for sycamore amyloplasts by the same authors [22]. In all cases, methylated sequences could be related to genes that were poorly expressed in the corresponding plastids. These observations led to the proposal that DNA methylation might play a key role in preventing transcription of individual genes in non-photosynthetic plastids [16, 22, 23]. A different mechanism has been proposed by Gruissem and his colleagues [6, 7, 12], following their discovery that transcriptional regulation played a very limited role in the control of gene expression during plastid development. Little or no transcriptional control has been observed in other systems [17] including chromoplast formation in red pepper fruits and sunflower petals [18].

We have investigated the patterns of methylation of both cp and crDNA from a local tomato cultivar that has a restriction map which closely resembles those reported earlier for other varieties [27–29]. Restriction patterns obtained after digestion of ptDNA with enzymes that are sensitive to the presence of methylated bases in their recognition sequences were similar for both types of plastids. Hybridization of the restriction fragments against a number of probes representing about 85% of the plastid genome also failed to reveal any difference, indicating that tomato ptDNA does not undergo methylation detectable by these procedures in the course of chromoplast formation.

Experimental procedures

Isolation of plastids and DNAs

Green leaves (100 g) and fully ripened red fruits (300 g) of multiple tomato plants (*Lycopersicon esculentum* cv. Platense) were used throughout the investigation. Plastids were isolated using the discontinuous sucrose gradient centrifugation technique. The method of Bathgate *et al.* [1] was employed for chloroplasts and that of Camara *et al.* [4] for chromoplasts. In both cases, the plastid bands were collected from the lower sucrose interphases, diluted to about 0.5 M sucrose by the slow addition of 50 mM Tris-HCl pH 7.5 and finally recovered by centrifugation for 10 min at 2500 *g*.

The plastid pellet was resuspended in 1–2 ml of 50 mM Tris-HCl pH 8.0, containing 400 mM sucrose, 7 mM EDTA, 0.1 mg/ml ribonuclease A and 2% w/v sodium sarcosinate. After 1 h at room temperature, proteinase K was added to a final concentration of 0.5 mg/ml and incubation prolonged for a additional hour. The preparation was extracted once with phenol, three to four times with phenol/chloroform and at least three times with water-saturated ether.

Plastid DNA was collected by ethanol precipitation and finally resuspended in 50 μl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The yields of cp- and crDNAs were about 30–40 μg per 100 g of leaf tissue and 10–15 μg per 100 g of pericarp tissue, respectively. Nuclear DNA was isolated from tomato leaves essentially as described by Watson and Thompson [39] except that nuclear extracts were treated with 0.1 mg/ml RNase A prior to proteinase K digestion and one CsCl was therefore omitted.

Digestion of plastid DNA and Southern transfer

Chloroplast or chromoplast DNAs (1–2 μg) were digested by various restriction endonucleases (about 10 units each) for 3 h in a reaction volume of 50 μl as specified by the suppliers. Restriction fragments were separated by electrophoresis on

1% agarose gels in 1 × TAE (40 mM Tris-acetate pH 8.0, 1 mM EDTA) and then transferred overnight to nitrocellulose filters (Hybond, Amersham) using 10 × SSC as transfer buffer [19].

Hybridization experiments

Hybridization probes were prepared by digestion of tobacco chloroplast DNA with *Pst* I and *Xho* I, followed by cloning of the fragments into compatible sites of pUC9. Plasmids were isolated by the alkaline lysis method [19] and labelled with [α-^{32}P]dATP by nick translation [19]. Vector pUC9 did not hybridize with plastid DNA, therefore labelling of the probes was carried out without digesting the cpDNA inserts from the recombinant plasmids. A detailed description of the probes employed is given in Table 1. Blotted filters were prehybridized at 57–60 °C for 3 h in plastic sealed bags containing 10–20 ml of 4 × SSC, 1 × Pi/PPi (0.05% NaPPi, 0.016 M sodium phosphate pH 7.0), 1 × Denhardt's solution [19], 0.2% w/v non-fat powder milk and 20 μg/ml denatured salmon sperm DNA, followed by hybridization at the same temperature for 24 h with 0.2 ml/cm^2 of prehybridization solution supplemented with 0.1% SDS, 10 mM EDTA and the [^{32}P]- labelled probes (10^6–10^7 cpm). Filters were washed (5 times for 15 min each at 45 °C in 3 × SSC, 1 × P/PPi and 0.1% SDS), and exposed to Kodak XAR-5 film for a minimum of 24 h.

Determination of DNA base composition

About 20 μg of plastid, nuclear or standard DNAs were hydrolyzed in sealed glass tubes with 90% (v/v) formic acid (1 μl/μg of DNA) at 175 °C for 30 min [22] and subjected to base composition analysis by reversed-phase HPLC, using a Konik HPLC 500-A system. Samples were applied onto a C18 reversed-phase column (Spherisorb S5 ODS2, PS, United Kingdom) and eluted with 10 mM NaH_2PO_4, pH 3.35, 2.4% (v/v) acetonitrile at a flow rate of 1 ml/min. Bases were monitored at 254 nm with a Gilson Holochrome Spectrophotometer and the peaks integrated in a Spectra-Physics SP4290 (San Jose, CA). Standards were from Sigma (St. Louis, MO).

Results and discussion

Restriction and gene maps of tomato plastid DNA

Construction of a restriction map of tomato chromoplast DNA was accomplished for the endonucleases *Kpn* I, *Sal* I, *Pvu* II and *Pst* I. *Bam* HI, which gives a larger number of smaller fragments, was also incorporated, thus conferring finer resolution on the map. Restriction fragments were oriented using the Hutchinson procedure and data from hybridization of single and double digested fragments against heterologous tobacco cpDNA probes, as described in Fig. 1. The loca-

Table 1. Probes from tobacco chloroplast genes used for hybridization experiments with restriction fragments from tomato plastid DNA. Naming of the genes is given according to Shinozaki *et al.* [34].

Probe	Size (kbp)	Genes present
Pst 2	21.13	rbcL, psaB, A, psbG, atpE, B, rps4, 14, trns, ORF62, 158
Pst 3	20.65	rbcL, petA, B, psbF, E, B, H, rpl33, 20, rps18, 12, trns, ORF512, 184, 229, 103
Pst 4b	19.35	psbA, atpA, F, H, I, rpoC, rps16, 2, trns
Pst 5	9.03	rpoC, B, trn
Pst 6a, b	7.42	rpl2, 23, trn, ORF581, 1708
Pst 8a, b	4.84	ndhB, rps7, trn, ORF115
Pst 9	3.87	petD, rpoA, rps11, 8, rpl14
Pst 10	2.74	rpl16, 22, rps3
Pst 11	2.25	psbA, rpl2, trn
Pst 12	1.45	rps19, rpl2
Pst 14	1.13	psbC, trn, ORF105
Xho 4a, b	11.93	rps7, 12, rRNA16S, trns, ORF1708, 115, 131
Xho 6	9.35	ndhF, rRNA23S, 4.5S, 5S, trns, ORF350, 313
Xho 12a, b	3.06	rRNA23S, trns
Xho 17a, b	0.81	rRNA23S

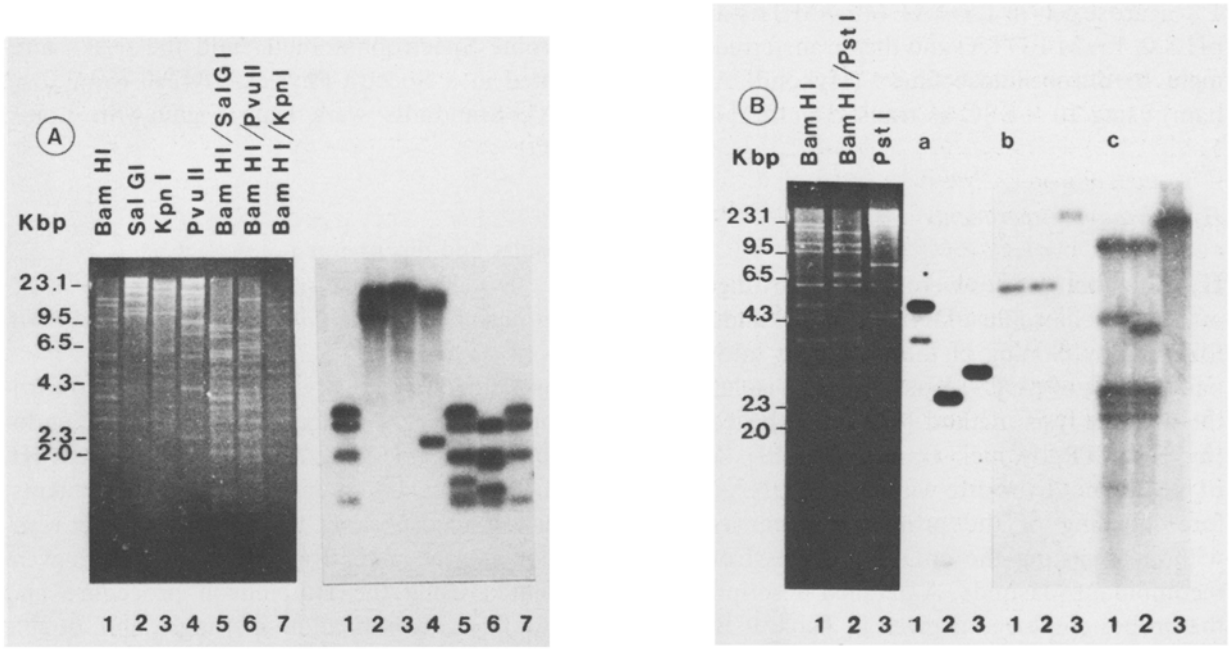

Fig. 1. Southern blot hybridization analysis of tomato chromoplast DNA against tobacco cpDNA probes. A. The DNA (2 μg) was digested with the enzymes indicated above the lanes of the ethidium bromide-stained gel (left side) and then transferred to nitrocellulose filters and probed with *Xho* 4. The resulting autoradiogram is shown on the right side. B. DNA (2 μg) was single- and double-digested with *Bam* HI and *Pst* I and electrophoresed on 1% agarose in the presence of ethidium bromide. The left side shows the ethidium bromide-stained gels and the right side autoradiograms of the hybridizations of the same DNA fragments probed with *Pst* 10 (a), *Xho* 17 (b) and *Pst* 2 (c). DNA sizes in kilobase pairs (kbp) are shown at the extreme left.

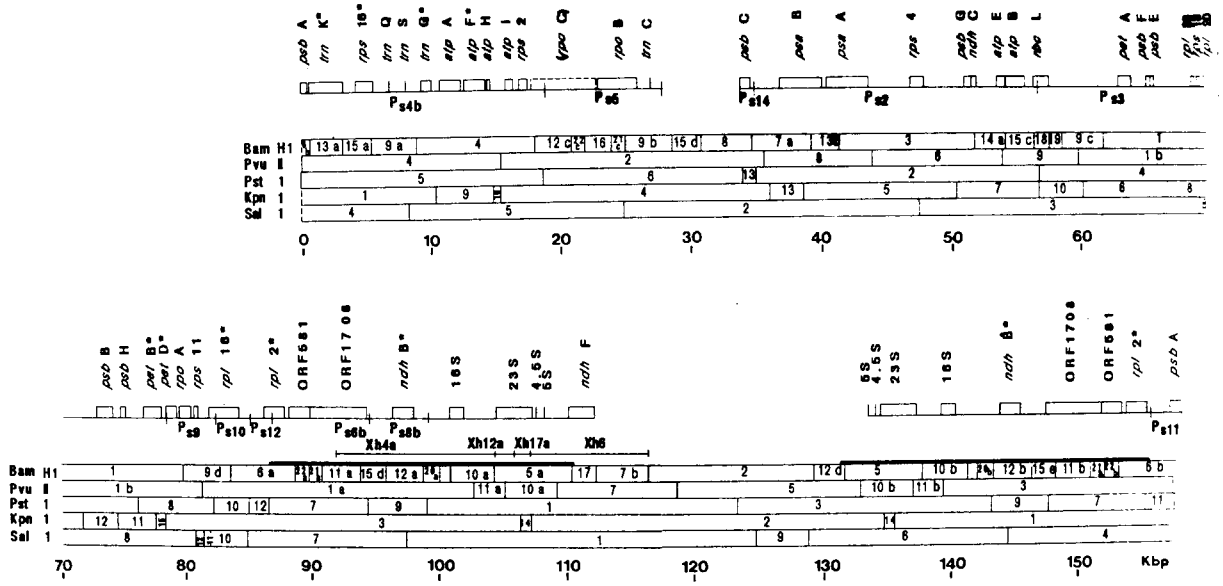

Fig. 2. Physical and gene maps of tomato chromoplast DNA. The maps are presented in linear forms. *Bam* HI, *Pst* I, *Kpn* I, *Pvu* II and *Sal* I restriction sites are indicated. Inverted repeats are shown in bold lines. The upper part represents the gene map, with the genes indicated within the tobacco cpDNA probes and named according to Shinozaki *et al.* [34]. Asterisks on some tobacco genes indicate that they contain introns [34].

tions of many genes and open reading frames on the tomato chromoplast chromosome were determined by this procedure. A list of the probes used, together with their gene content, is given in Table 1. They encompass about 85% of the tobacco chloroplast genome [34]. The resulting gene map is shown in Fig. 2. Identical results were obtained when chloroplast DNA was used instead of crDNA (not shown). The physical and gene maps of Fig. 2 agree well with those reported earlier [27–29, 31]. The *Eco* RI-generated restriction pattern was also similar to a previously published one [15]. The gene locations and most of the restriction sites are well conserved with respect to those of other members of the Solanaceae and the majority of vascular plants [10, 25, 34].

Methylation patterns of tomato plastid DNA

DNA methylation is known to play a key role in regulating gene expression. The rate of transcription of several plant and vertebrate nuclear genes has been shown to be affected by the levels of DNA methylation [37, 38]. In the case of plastids, methylated DNA is usually not found in chloroplasts from C3 plants, but recent results indicate that plastid DNA from sycamore amyloplasts, tomato chromoplasts, and maize mesophyll cell chloroplasts show higher levels of methylation than the corresponding control chloroplasts [16, 22–24]. The patterns of methylation correlated well with the steady-state amounts of specific gene transcripts, suggesting that the lower mRNA levels were due to an impairment of the transcription rate of individual genes. However, studies in a number of systems indicate that transcriptional regulation plays a very limited role in plastid differentiation [6, 7, 12, 18, 21] and that gene expression is mostly controlled by the post-transcriptional processing and/or stability of constitutively synthesized plastid transcripts [12, 35].

We have examined the patterns of base methylation of the plastid genome of cv. Platense, which is structurally similar to those described in the literature [15, 27, 28, 31]. Chloroplast or chromoplast DNAs were completely hydrolyzed [22] and subjected to base composition analysis by reversed-phase HPLC (Fig. 3). Elution profiles of the acid hydrolysates yielded the four normal

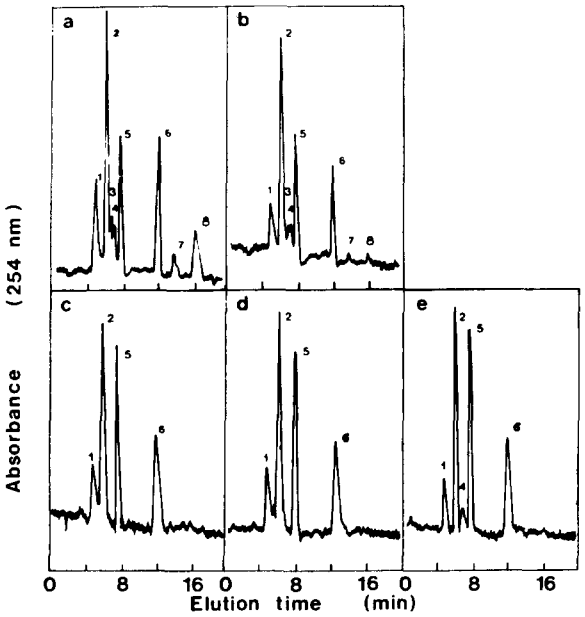

Fig. 3. Reversed-phase HPLC profiles of acid hydrolysates of standard bases and tomato nuclear and plastid DNA. (a) A mixture of the four major bases (~100 ng of each) and four modified bases (~40 ng of each), chosen among those reported to be the most abundant in non-photosynthetic plastid DNA [16, 22, 23]. Each base was independently subjected to formic acid treatment as described in the Experimental section. (b–e) Acid hydrolysates corresponding to 0.6 μg of leaf nuclear DNA (b), chloroplast DNA (c) and chromoplast DNA (d, e). In (e) the crDNA hydrolysate was supplemented with 10 ng of 5-methylcytosine prior to chromatography. Absorbance scales (arbitrary units) were the same in a–e. All other conditions are described under Experimental Procedures. Peaks are identified as follows: 1, cytosine (C); 2, guanine (G); 3, 3-methylcytosine (3-MeC); 4, 5-methylcytosine (5-MeC); 5, thymine (T); 6, adenine (A); 7, 7-methylguanine (MeG) and 8, N6-methyladenine (MeA). Base compositions averaged from two separate experiments were (mol %): nuclear DNA (b): C (17.1), G (22.0), 3-MeC (2.5), 5-MeC (5.2), T (26.7), A (24.9), MeG (1.2), MeA (0.5); chloroplast DNA (c): C (18.1), G (17.0), T (31.8), A (33.2); chromoplast DNA (d): C (17.5), G (17.9), T (33.0), A (31.6); chromoplast DNA plus 5-methylcytosine (e): C (15.3), G (16.5), 5-MeC (4.2), T (32.6), A (31.5). Values were calculated by standardization of the elution peak areas against those of the corresponding standard bases.

bases (Fig. 3c and d) without significant differences between cp- and crDNA. Use of the column and conditions described [22] gave essentially the same results but with less resolution between cytosine and 3- or 5-methylcytosine (data not shown). Addition of 10 ng of 5-methylcytosine to a crDNA hydrolysate gave a clearly distinct peak (Fig. 3e) which accounted for about 4% of the total bases present. In contrast, leaf nuclear DNA showed a variety of modified bases (comprising about 10% of the total DNA, Fig. 3b). The G + C content of tomato leaf nuclear DNA was 48% and that of tomato plastid DNA was 35% (Fig. 3).

When the extent of methylation of a given genome is too low to be reflected in its base composition the presence of rare modified bases can be detected and mapped by isoschizomer analysis. This procedure only identifies methylated nucleotides located within the recognition sequences of the sensitive enzymes. In spite of its shortcomings, the method has been widely employed to evidence this type of modification in

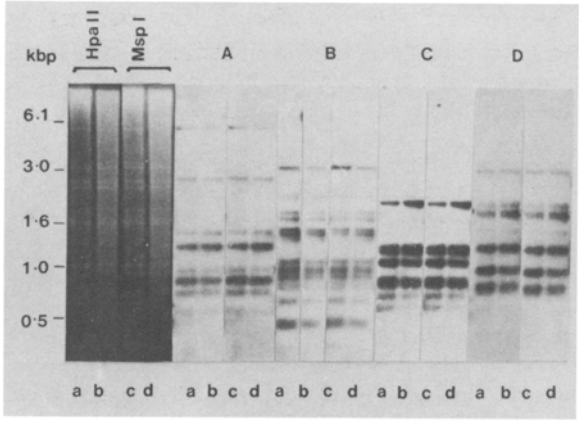

Fig. 4. Methylation analysis of plastid DNA at *Msp* I/*Hpa* II sites. Equivalent amounts (2 μg) of chloroplast (lanes a and c) and chromoplast DNAs (lanes b and d) were digested with *Hpa* II and *Msp* I (15 units each) for 3 h and subjected to 1.2% agarose gel electrophoresis. Fragments were then transferred to nitrocellulose filters and hybridized. The ethidium bromide-stained gel is shown on the left side, whereas the right side displays typical autoradiograms obtained after hybridization of the blotted filter against probes *Xho* 6 (A), *Pst* 3 (B), *Pst* 6 (C) and *Pst* 2 (D) from tobacco cpDNA (Table 1). DNA sizes (kbp) are shown at the extreme left.

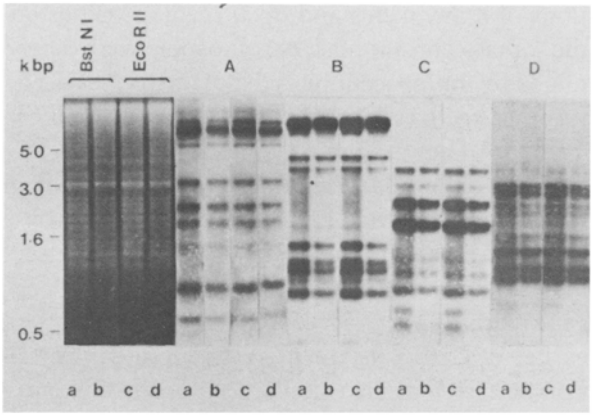

Fig. 5. Methylation analysis of tomato plastid DNA at *Bst* NI/*Eco* RII sites. Chloroplast (lanes a and c) and chromoplast (lanes b and d) DNAs were digested with *Bst* NI and *Eco* RII, transferred to nitrocellulose filters and hybridized against probes *Xho* 6 (A), *Pst* 3 (B), *Pst* 6 (C) and *Pst* 2 (D) essentially as described in the legend to Fig. 3. Left side, gels stained with ethidium bromide; right side, autoradiograms.

a number of genomes including plastid and nuclear ones [22–24]. *Pst* I, for instance, cleaves the sequence 5′-CTGCAG-3′ only when the cytosines are unmethylated. The enzyme has been successfully used to reveal extensive DNA methylation in plant nuclei, and therefore estimate the degree of nuclear DNA contamination in ptDNA samples [3]. *Pvu* II and *Sal* I show essentially the same behavior [26]. Digestion of ptDNA with these endonucleases, however, gave identical restriction and hybridization patterns for both cp- and crDNAs (compare the patterns of Fig. 1. with those of Gounaris *et al.* [10]).

Msp I and *Hpa* II cleave the sequence 5′-CCGG-3′, but double methylation of the cytosines prevents *Hpa* II digestion. The restriction patterns obtained with these two enzymes looked identical for both cp- and crDNA (Fig. 4). *Eco* RII, an isoschizomer of *Bst* NI, is unable to digest the 5′-CC(A/T)GG-3′ sequence when the internal cytosine is methylated. Our samples gave essentially the same patterns irrespective of the enzymes and DNAs used (Fig. 5). Finally, *Sau* 3AI cleaves the 5′-GATC-3′ sequence, when the adenine is methylated and the cytosine is unmethylated, whereas its isoschizomer *Mbo* I

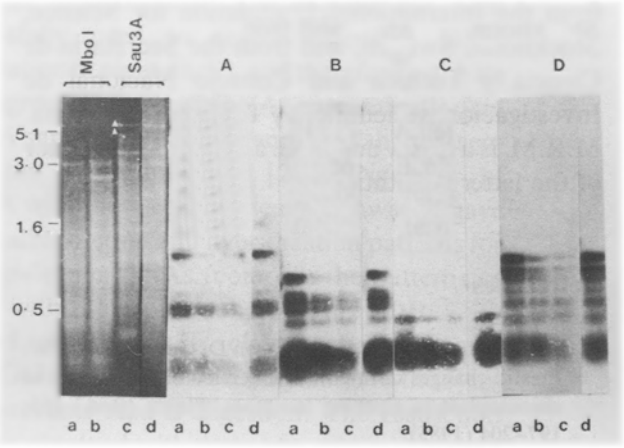

Fig. 6. Methylation analysis of tomato plastid DNA at *Mbo* I/*Sau* 3AI sites. Chloroplast (lanes a and c) and chromoplast (lanes b and d) DNAs were digested with *Mbo* I and *Sau* 3AI, blotted onto nitrocellulose filters and finally hybridized against probes *Xho* 6 (A), *Pst* 3 (B), *Pst* 6 (C) and *Pst* 2 (D). Experimental conditions were essentially those of Fig. 4. Ethidium bromide-stained gels are shown on the left side and autoradiograms on the right side. Different cleavage sites are indicated by white triangles on lanes c and d of the ethidium bromide-stained gels, and correspond to bands of 5.5 and 4.6 kbp.

shows the converse activity. This was the only pair for which we could detect the presence of 5-methylcytosine in the target sequence (Fig. 6). However, this methylation appears not to be developmentally regulated since both cp- and crDNAs show the same length of polymorphisms for *Sau* 3AI (Fig. 6).

Full digestion of ptDNA was verified as follows. In a parallel assay a mixture of ptDNA and an internal control (usually pBR322 or phagemid Blue Scribe) was digested with the corresponding enzymes and the resulting fragments were analyzed by ethidium bromide staining and hybridizations (data not shown).

Restriction fragments obtained with all the enzymes were hybridized with the probes of Table 1. Typical experiments are displayed in Fig. 4–6, in which we illustrate results obtained with four of the bigger probes. No chromoplast-specific length polymorphism could be detected with any of the probes, even when they contained genes with very different expression behaviors in response to chromoplast differentiation: some genes are expressed only or preferentially in chloroplasts, whereas others are specifically triggered in chromoplasts [20]. It might be speculated that the lack of crDNA methylation in cv. Platense could result in enhanced amounts of mRNA in chromoplasts. However, northern analysis of total RNA from mature fruits [20] indicated the same general patterns for transcript levels that were described by Piechulla *et al.* [31, 32].

Evaluation of nuclear material contamination in our ptDNA preparations was carried out by hybridizing the blotted filters of Fig. 4–6 against a spinach cDNA probe coding for the nuclear-encoded light-harvesting chlorophyll *a*/*b*-binding protein (*cab*). No detectable signals could be observed, even after prolonged exposure (not shown). Moreover, plant nuclear DNA is very imperfectly digested by *Pst* I, *Pvu* II or *Sal* I [3, 26], and would have been conspicuous in agarose gels as a slowly migrating broad band, which is not the case (Fig. 1). In any event, contamination with nuclear (methylated) DNA would be a serious drawback in an argument *in favour* of ptDNA methylation, but our results indicate quite the opposite situation. Still, we can not rule out the existence of methylated bases in crDNA located at important regulatory sites different from the target sequences of the enzymes we used. A proportionally small number of modified bases in a whole genome would impair the expression of many genes.

We do not know the reasons for the discrepancy between our results and those of Ngernprasirtsiri *et al.* [16, 23], but it might reflect genetic differences in the cultivars employed. Indeed, the ptDNA used in ref. 23 is somehow 15 kb shorter (141 kb against 156 kb) than the reported size for tomato, an observation made by the authors themselves. It is therefore possible that cv. Firstmore might be different not only in its chromosomal arrangement but also in displaying a particular mechanism of gene inactivation by methylation not present in other varieties or species. The question can be raised whether different mechanisms of gene expression control might exist among different varieties.

There are of course many cultivars of tomato and it would be more likely to conceive a basic mechanism governing gene expression for them all. However, our results indicate that methylation sites in cv. Platense, if they exist, are located differently from those reported to be functionally important in cv. Firstmore. Actually, the latter cultivar has undergone a major deletion of its plastid genome, a process far more drastic than the change in methylation behavior. It might be interesting to compare the ptDNA restriction and gene maps of both varieties, in order to determine which regions of the plastid genome are absent in cv. Firstmore.

The arguments discussed here relate to the controversy among different groups concerning the role of transcriptional control on plastid differentiation [6, 7, 12, 16–18, 21–24]. Transcriptional regulation, as well as its relation to DNA methylation, is well documented for plant nuclei [8, 24]. In view of the seemingly wide distribution of DNA methylation among non-photosynthetic plastids, it is very important to distinguish the rule from the exception in the different behaviors of cvs. Firstmore and Platense, and whether cultivar variabilities could account for such major changes in the regulation of gene expression. Within this context, we have analyzed crDNA isolated from cultivars Raci53, CalJ, UC82 and Rossol, also available in our market. Both restriction and isoschizomer analyses yielded similar results as those obtained with cv. Platense (not shown) although we did not carry out systematic hybridizations with all the probes of Table 1. Incidentally, use of isoschizomers also failed to reveal DNA methylation in spinach root amyloplasts [7]. Further work will be necessary to elucidate this point.

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