Volume 275, number 1,2, 102-106 FEBS 09139 November 1990

DNA polymerase activity of tomato fruit chromoplasts

Esteban C. Serra and Nestor Carrillo

Departamento de Ciencias Biológicas, Area Biología Molecular, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531 (2000) Rosario, Argentina

Received 29 June 1990; revised version received 5 October 1990

DNA polymerase activity was measured in chromoplasts of ripening tomato fruits. Plastids isolated from young leaves or mature red fruits showed similar DNA polymerase activities. The same enzyme species was present in either chloroplasts or chromoplasts as judged by pH and temperature profiles, sensitivities towards different inhibitors and relative molecular mass (M_r 88 kDa). The activities analyzed showed the typical behaviour of plastid-type polymerases. The results presented here suggest that chromoplasts maintain their DNA synthesis potential in fruit tissue at chloroplast levels. Consequently, the sharp decrease of the plastid chromosome transcription observed at the onset of fruit ripening could not be due to limitations in the availability of template molecules. Other mechanisms must be involved in the inhibition of chromoplast RNA synthesis.

DNA polymerase; Chromoplast; Plastid gene expression; Fruit ripening; Tomato; *Lycopersicon esculentum*

1. INTRODUCTION

The ripening process in tomato fruits is characterized by the disappearance of chloroplasts (cp) and the formation of non-photosynthetic chromoplasts (cr). Cr differentiation involves a number of morphological and biochemical changes, including starch degradation, breakdown of chlorophyll and thylakoid membranes, and synthesis and accumulation of carotenoids [1]. In addition, photosynthetic proteins and their corresponding mRNAs are absent or greatly diminished [2,3]. These changes in the patterns of gene expression are not due to major recombinational arrangements of the pt genome, since restriction patterns of cp- and crDNAs are identical in tomato [2,4,5], and other species [6,7].

Two different processes contribute to regulate gene expression during chromoplast biogenesis: (a) an overall decrease (5–10-fold) of the transcriptional activity of chromoplasts as compared to chloroplasts ([8,9], Marano and Carrillo, unpublished), that affects most plastid genes to the same extent [10] and (b) a specific impairment in the expression of individual genes in non-photosynthetic plastids, a phenomenon that has been attributed to transcription initiation [9], transcript processing and/or stability [8,10] and translational control [11].

Correspondence address: N. Carrillo, Departamento de Ciencias Biológicas, Area Biología Molecular, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531 (2000) Rosario, Argentina

Abbreviations: cr, chromoplast; cp, chloroplast; pt, plastid; araCTP, cytosine β-D-arabinofuranoside 5′-triphosphate; bp, base pairs; NEM, *N*-ethylmaleimide; Tris, tris (hydroxymethyl)aminometane; MES, 2-(*N*-morpholino) ethanosulfonic acid; MOPS, 3-(*N*-morfolino)propanesulfonic acid.

Variation in DNA content might influence the former activity (overall transcription rate), particularly if plastid transcription is template limited. The amount of DNA in the organelle varies considerably among different tissues [12] although no clear correlation could be established between the number of DNA molecules and gene expression of the plastid [8,13]. On the other hand, little is known concerning DNA replication in non-photosynthetic plastids. A chloroplast DNA polymerase has been isolated from pea leaves [14]. The enzymes from pea, petunia and maize chloroplasts are monomeric proteins of about 90 kDa which show replicase activity in vitro with cpDNA templates that contain putative replication origins [15–17].

In the present paper, we have measured DNA polymerase activity in red fruit chromoplasts and found that it did not differ significantly from the corresponding leaf chloroplast level. We also report some structural and kinetic properties of the chromoplast enzyme.

2. MATERIALS AND METHODS

2.1. *Plastid Isolation*

Tomato (*Lycopersicon esculentum* L. cv Platense) plants were grown under greenhouse conditions without additional light and temperatures between 20 and 25°C. Young leaves (1–1.5 cm) from 30 day old plants and fully ripened red fruits were used throughout the experiments. About 300 g of pericarp tissue or 100 g of leaves were sterilized with 80% ethanol, extensively washed with ice-cold sterile distilled water and finally homogenized with a polytron in a grinding medium containing 50 mM Tris-HCl pH 8.0, 0.4 M sucrose, 1 mM EDTA, 1 mM 2-mercaptoethanol, 2 mM sodium ascorbate and 0.3% w/v polyvinylpyrrolidone. Homogenates were filtered through 4 layers of cheesecloth and two layers of miracloth and then centrifuged for 15 min at 3000 × *g*. Pellets were resuspended in a small volume of grinding medium and subjected to discontinuous sucrose gradient centrifugation. The procedure of Bathgate et al. [18] was used for chloroplasts and that of Camara et al. [19] for chromoplasts. In both cases, plastid bands were collected from the lower sucrose interphases

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

and diluted to 0.5 M sucrose by the slow addition of 50 mM Tris-HCl pH 7.5. Plastids were collected by centrifugation (10 min at 2500 × g) and finally resuspended in a small volume of grinding medium. Cytochrome c oxidase activity [20] was less than 0.01 U/10^7 plastids, indicating that the preparations were largely free of contaminating mitochondria. All previous manipulations were carried out at 4°C. Plastid concentrations were determined by counting on a modified Neubauer camera.

DNA content in plastids was estimated essentially as described by Baumgartner et al. [13], using dot blot hybridization of crude plastid DNA preparations with tobacco chloroplast fragment *Pst*I-3 [21], which is well conserved in tomato [5].

2.2. *DNA polymerase assay*

Polymerase activity was assayed essentially as described in [17], using 5 μl of the plastid extracts as enzyme source and 5 μg of either DNAse-activated salmon sperm DNA or plasmid pZmcEcox [17] as template. Acid insoluble radioactivity was determined according to Tewari [22]. When measuring the effect of pH on the activity, the same reaction medium was employed, except that the Tris buffer used in the normal assay (50 mM, pH 7.6) was replaced by a mixture of 20 mM each of MES, MOPS and Tris. The pH was varied between 5.5 and 9.0, without changing the chemical nature of the buffers.

2.3. *Analytical procedures*

The polypeptide composition of chloro- or chromoplast lysates was determined by polyacrylamide gel electrophoresis [23]. Separating gels used for activity staining [24] contained 150 μg/ml of activated DNA. Preparation of the samples and conditions for electrophoresis and DNA polymerase staining were essentially those of ref. [17]. Random primer labeling [25] of *Pst*I-3 and hybridizations [26] were carried out following published procedures.

2.4. *Materials*

Activated salmon sperm DNA was prepared according to Tewari [22]. [α-^{32}P]dATP was from New England Nuclear (Ducilo, Argentina) and nitrocellulose (0.45 μm) from Amersham (ERM, Argentina). All other reagents were of analytical grade.

3. RESULTS

3.1. *Plastid DNA polymerase activity*

The DNA replication capacity of plastids isolated from tomato leaves and mature red fruits was estimated by measuring DNA polymerase activity of plastid lysates. Table I shows polymerase activity of chromoplast extracts as compared to chloroplasts. As reported by McKown and Tewari [14], plastid lysates show considerable endogenous activity, which was considered unsuitable for comparison purposes due to uncertainties about the amount of prDNA templates. After degradation of endogenous DNA [14] the repair activity of the extracts was assayed using DNAse-treated salmon sperm DNA as template (Table I). In addition, chromoplast lysates were able to replicate on native plasmid pZmcEcox (data not shown), which contains a putative replication origin for maize cpDNA and is a preferred template for in vitro DNA synthesis by both pea and maize cpDNA polymerase [15,17]. Polymerase activity with both endogenous and exogenous substrates was high in chromoplasts (Table I). The results indicate that chromoplasts, although showing low transcription rates when compared to leaf chloroplasts [8,9], keep their DNA synthesis potential at chloroplast levels.

Table I

DNA polymerase activity in plastids of tomato young leaves and mature red fruits

Plastid type	dNMP incorporated[a] (pmol)	
	Endogenous template[b]	Activated DNA template[c]
Chloroplasts	0.12 ± 0.01	0.30 ± 0.01
Chromoplasts	0.10 ± 0.01	0.28 ± 0.01

[a]Nucleotides incorporated by 10^7 plastids during 30 min of reaction. Average of 6 experiments. [b]DNA polymerase activity was assayed as described in section 2, omitting the addition of DNAse-activated salmon sperm DNA. [c]Endogenous prDNA was eliminated as described by McKown and Tewari [14] until activity of the extracts in the absence of endogenous template was negligible. DNA synthesis was then assayed using 5 μg of activated salmon sperm.

Relative amounts of DNA per plastid, measured by dot blot hybridization with tobacco chloroplast *Pst*I-3 fragment [21] as probe, did not change significantly between leaf chloroplast, green fruit chloroplasts and mature red fruit chromoplasts (data not shown), although more careful experiments will be necessary to estimate the absolute average number of copies per plastid or cell.

3.2. *Comparison of plastid DNA polymerases*

Although the overall replication activity is virtually unchanged in fruit plastids, the possibility remains open that an altered or completely different enzyme is synthesized in fruits. In addition, possible contaminating activities from nuclear and/or mitochondrial origins must be ruled out to reach sound conclusions. To test for these possibilities we further characterized the chromoplast DNA polymerase and compared its catalytic and structural properties with those of the chloroplast enzyme. The polymerases from both sources showed broad pH optima (between pH 7.5 and 8.5, Fig. 1A) in good agreement with previous reports [14]. Under standard conditions (pH 7.6) the maximal activity was obtained at 30°C (Fig. 1B). Above that temperature the enzymes were progressively denatured in an irreversible way (data not shown).

The polymerases were highly sensitive to *N*-ethylmaleimide and araCTP. Ethanol and ethidium bromide were also strong inhibitors. Aphidicolin, on the other hand, had no effect on the enzyme activity, and ddTTP, when used at a 1:1 molar ratio with respect to dTTP, was a rather poor inhibitor (Table II). The previous results agree well with the general properties reported for plastid-type DNA polymerases [14,16,17].

Activity staining of polyacrylamide gels was used to estimate the molecular mass of the polymerases contained in both plastid lysates [26]. This method allows determination of individual proteins in crude extracts, provided that the catalytic activity is confined to monomeric subunits which can be renatured after SDS

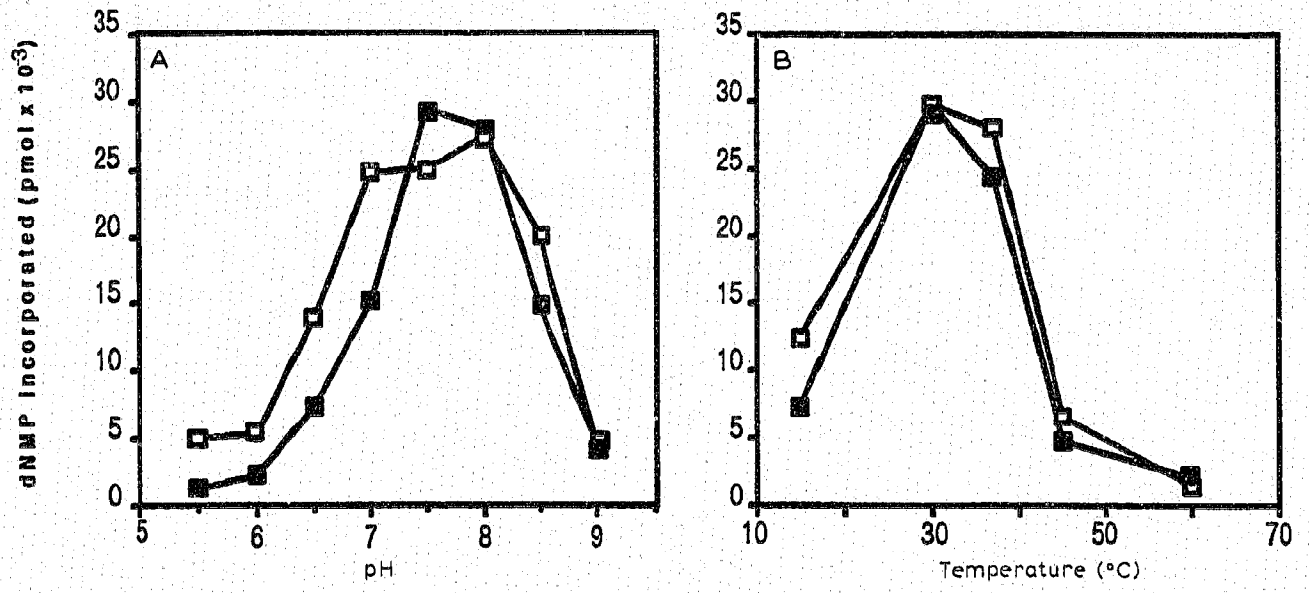

Fig. 1. pH (A) and temperature (B) profiles of the DNA polymerase activities from tomato plastid lysates. In (A) the reaction medium contained a mixture of 20 mM each of MES, MOPS and Tris and the pH was varied between 5.0 and 9.0. In (B) the reaction medium was 50 mM Tris-HCl pH 7.6. All other experimental conditions are described in section 2, (- □ - □ -) chloroplasts, (- ■ - ■ -) chromoplasts.

electrophoresis. A single DNA polymerase band fulfilling the previous conditions was identified in crude extracts from either chloroplasts or chromoplasts (Fig. 2A). In both cases the relative molecular mass was 88 kDA (Fig. 2B), which is in the range of previously reported values [14,16,17].

4. DISCUSSION

Fruit maturation involves, among several other changes, the conversion of photosynthetic chloroplasts into non-photosynthetic carotene-rich chromoplasts, a transition after which the overall transcription activity per individual plastid sharply decreases [8,9] and many cp-specific peptides and their mRNAs become undetectable. Our results indicate that the decrease in transcription is not likely to be associated with shortage of template availability in chromoplasts, since relative ptDNA average amounts and plastid DNA replication capacity remain fairly constant in both leaf chloroplasts and red fruit chromoplasts.

Table II

Inhibitory effects on tomato plastids DNA polymerase

Inhibitors	dNMP incorporated[a] (pmol)	
	Chloroplasts	Chromoplasts
None	0.31 (100)	0.29 (100)
1 mM NEM[b]	0.06 (19)	0.10 (34)
10% ethanol	0.02 (6)	0.03 (11)
0.1 mM aphidicolin	0.30 (97)	0.28 (96)
25 μM ethidium bromide	0.02 (6)	0.04 (14)
10 mM araCTP	0.01 (3)	0.05 (17)
50 μM ddTTP	0.20 (65)	0.22 (76)

[a]Nucleotides incorporated by 10^7 plastids during 30 min of reaction. Average of 4 experiments. Numerals between parentheses indicate percentage of residual activity. [b]Plastid lysates were pre-incubated with *N*-ethylmaleimide at 25°C for 15 min in the reaction buffer (no nucleotides present). All other reagents were assayed during the reaction, without preincubation.

Chloroplast DNA polymerases are very minor peptides in leaf tissue [14,17], which has precluded so far the preparation of specific antibodies. In the absence of immunological methods of detection, we had to rely on activity measurements to estimate polymerase levels. Overall activity with both endogenous (ptDNA) or exogenous templates was very similar in both plastid lysates (Table I).

In order to determine whether the same polymerase(s) were present in the two types of lysates, a number of kinetic measurements were carried out. Polymerases from both types of plastid lysates showed similar pH and temperature profiles (Fig. 1) and the same sensitivity towards inhibitors (Table II).

The nuclear α-like DNA polymerases are strongly inhibited by aphidicolin (see [27] and refs. therein). By contrast, plastid lysates showed less than 5% inhibition by this reagent (Table II). The other nuclear activity, chromatin-bound β-like polymerase, is highly resistant to NEM and araCTP [27], whereas plastid enzymes were drastically inhibited (Table II). Our plastid preparations were substantially free from mitochondrial contamination (section 2) and their polymerase activities were only moderately affected by ddTTP, while mitochondrial polymerases show exquisite sensitivity towards this nucleotide analog [27,28]. The previous

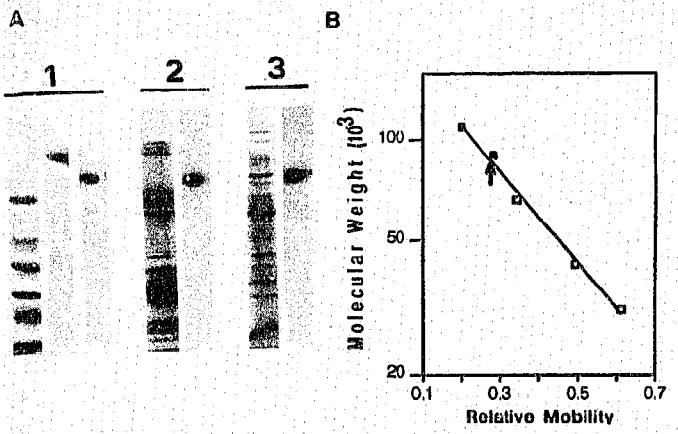

Fig. 2. Polypeptide composition of tomato plastid lysates as analyzed by SDS polyacrylamide gel electrophoresis. Experimental conditions are described in section 2. (A) Lane 1, molecular mass standards; lane 2, chloroplast extract; lane 3, chromoplast lysate. The left half member of each pair shows polypeptide patterns in the gel visualized by Coomassie brilliant blue staining. The right-hand members are autoradiograms showing the location of DNA synthetic activity of several DNA polymerases (lane 1) or plastid lysates (lanes 2 and 3). (B) Determination of the approximate molecular mass of tomato plastid DNA polymerase in the activity gels. Two types of molecular mass standards were used: Sigma Dalton Mark VII-L (Sigma Chemical Co. St. Louis, MO) stained with Coomassie blue (- □ - □ -): bovine serum albumin, 66 kDa; ovalbumin, 43 kDa; glyceraldehyde-3-P-dehydrogenase, 36 kDa, and polymerase standards revealed by activity staining (- ■ - ■ -): *Escherichia coli* DNA polymerase I, 110 kDa and pea chloroplast DNA polymerase, 90 kDa. The arrow indicates relative mobility corresponding to cp- and crDNA polymerases.

results argue against any significant nuclear and/or mitochondrial contaminating activities. In general, DNA polymerase present in chromoplast extracts showed typical properties of pt-like enzymes [14,16,17], including the apparent molecular mass of 88 kDa (Fig. 2). Besides the plastid enzyme, no other plant DNA polymerase described so far has a molecular mass near this value [27]. Activity gel electrophoresis of plant extracts indicated the presence of two polymerase bands with apparent molecular masses of 70 and 110 kDa [27]. As judged by the assays carried out in this work, the same molecular species of DNA polymerase was present in either chloroplasts or chromoplasts.

The finding that ptDNA polymerase (most probably a nuclear encoded enzyme) is apparently synthesized in a constitutive manner is somehow surprising. Cell elongation ceases in the early stages of fruit development and the plastid content per cell does not appear to change thereafter [29]. Possibly DNA polymerase plays a 'housekeeping' role in chromoplasts, repairing damaged DNA molecules and/or replacing degraded ones, thus keeping the plastid DNA content high even after differentiation.

Since overall transcription does not seem to be regulated by fluctuations in replication capacity, RNA synthesis inhibition after chromoplast formation might be due to limiting RNA polymerase levels or to changes in plastid template that could affect transcriptional competence. Both topological changes [30] and DNA methylation [9] of pt DNA have been reported to affect transcription in chloroplasts and non-photosynthetic plastids. Work is now in progress to elucidate these questions.

Acknowledgements: We wish to thank Dr Eduardo Ceccarelli for critical reading of the manuscript and many additional helpful suggestions, and Drs Lawrence Bogorad and Alan Browdell, Harvard University, USA for the clone Ps-3. This work was supported by grants from the International Foundation for Science (IFS, Stockholm, Sweden, R.G. Agreement C/1354-1), Third World Academy of Science (TWAS, Trieste, Italy, RG BC 88-88) and Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET, Argentina). E.C.S. is a fellow and N.C. a Career Investigator of the latter Institution.

REFERENCES

[1] Grierson, D. (1986) Oxford Surv. Plant Mol. Cell Biol. 365–383.
[2] Piechulla, B., Chonoles-Imlay, K.R. and Gruissem, W. (1985) Plant Mol. Biol. 5, 373–384.
[3] Piechulla, B., Pichersky, E., Cashmore, A.R. and Gruissem, W. (1986) Plant Mol. Biol. 7, 367–376.
[4] Hunt, C.M., Hardison, R.C. and Boyer, C.D. (1986) Plant Physiol. 82, 1145–1147.
[5] Marano, M.R. and Carrillo, N. (1990) in: Progress Research in Photosynthesis (Baltschefsky, M. ed) vol. 3, pp. 865–868, Kluwer, Dordrecht.
[6] Gounaris, I., Michalowski, C.B., Bohnert, H.J. and Price, C.A. (1986) Curr. Genet. 11, 7–16.
[7] Hansmann, P. (1987) Z. Naturforsch. 42c, 118–122.
[8] Deng, X.-W. and Gruissem, W. (1987) Cell 49, 379–387.
[9] Kobayashi, H., Ngernprasirtsiri, J. and Akazawa, T. (1990) EMBO J. 9, 307–313.
[10] Gruissem, W. (1989) Cell 56, 161–170.
[11] Kuntz, M., Evrard, J.L., d'Harlingue, A., Weil, J.H. and Camara, B. (1989) Mol. Gen. Genet. 216, 156–163.
[12] Bendich, A.J. (1987) BioEssays 6, 279–282.
[13] Baumgartner, B.J., Rapp, J.C. and Mullet, J.E. (1989) Plant Physiol. 89, 1011–1018.
[14] McKown, R.L. and Tewari, K.K. (1984) Proc. Natl. Acad. Sci. USA 81, 2354–2358.
[15] Gold, B., Carrillo, N., Tewari, K.K. and Bogorad, L. (1987) Proc. Natl. Acad. Sci. USA 84, 194–198.
[16] de Haas, J.M., Kool, A.J., Overbeeke, N. and Nijkamp, H.J.J. (1987) Curr. Genet. 12, 377–385.
[17] Carrillo, N. and Bogorad, L. (1988) Nucleic Acids Res. 16, 5603–5620.
[18] Bathgate, B., Purton, M.E., Grierson, D. and Goodenough, P.W. (1987) Planta (Berlin) 165, 197–204.
[19] Camara, B., Bardat, F., Dogbo, O., Brangeon, J. and Moneger, R. (1983) Plant Physiol. 73, 94–99.
[20] Brambl, R. (1977) Arch. Biochem. Biophys. 182, 273–281.
[21] Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chungwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torasawa, K., Meng, B.J., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Tohdoh, N., Shimada, H. and Sugiura, M. (1986) EMBO J. 5, 2043–2049.
[22] Tewari, K.K. (1986) Methods Enzymol. 118, 186–201.
[23] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[24] Karawya, E., Swack, J.A. and Wilson, S.H. (1983) Anal. Biochem. 135, 318–325.

[25] Feinberg, A. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[26] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[27] Litvak, S. and Castroviejo, M. (1987) Mut. Res. 181, 81–91.
[28] Insdorf, N.F. and Bogenhagen, D.F. (1989) J. Biol. Chem. 264, 21491–21497.
[29] Coombe, B.G. (1976) Annu. Rev. Plant Physiol. 27, 507–528.
[30] Stirdivant, S.M., Crossland, L.D. and Bogorad, L. (1985) Proc. Natl. Acad. Sci. USA 82, 4886–4890.