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Plastid transcripts in chloroplasts and chromoplasts of *Capsicum annuum*

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Summary. Plastids from red and green fruits of the bell pepper, *Capsicum annuum* L., var. *Emerald Giant*, were examined for the presence of chromoplast-specific transcripts. Plastid DNA fragments produced by the restriction endonuclease *Pvu*II were individually cut into small pieces, resolved on agarose gels, blotted, and probed with labelled cDNAs prepared from total chloroplast or chromoplast RNA. Although many chloroplast transcripts were detected at high levels in the red fruits, we saw no consistent evidence of chromoplast-specific transcripts. In order to increase the signal-to-noise ratio, chromoplast cDNA probes were absorbed with an excess of chloroplast RNAs. We also examined in greater detail regions of the plastid genome thought to contain unidentified open reading frames. None of the Southern blots provided unambiguous evidence of chromoplast-specific transcripts. Taken together with other data, these results make it unlikely, but do not exclude the possibility that plastid genes contribute to chromoplast development.

Key words: Chromoplast – Pepper – RNA transcripts

Introduction

The differentiation of chloroplasts into chromoplasts in *Capsicum annuum* reveals a well defined sequence of events in which chlorophyll is degraded; the thylakoid system loses its grana, diminishes, and moves peripherally; dilated membranous and fibrous structures appear; the levels of carotenoids increase; and there is an appearance of new kinds of carotenoids, mainly epoxidated xanthophylls (Spurr and Harris 1968; Davies et al. 1970; Camara 1978; Camara and Monéger 1978; Camara and Brangeon 1981).

Patterns of inheritance of pigments in pepper fruits clearly indicate the involvement of nuclear genes in chromoplast development (Kormos and Kormos 1960; Hurtado-Hernandez and Smith 1985). Chromoplasts contain DNA that appears to be identical to cpDNA in *Narcissus pseudonarcissus* (Thompson 1980), tomato (Iwatsuki et al. 1985), and *Capsicum annuum* (Gounaris et al. 1986), but no maternally inherited characters affecting chromoplast development have been described. Indeed all of the genes identified in the chloroplast genome thus far code either for components of the photosynthetic apparatus or for components required for the transcription and translation of the plastid genome (cf. Shinozaki et al. 1986). Our objective is to identify and characterize genes regulating chromoplast development. At the outset, we thought it worthwhile to examine the possibility of chromoplast-specific genes in the plastid genome. If such genes occur, they could offer an important insight into the problem of chromoplast development.

Since the photosynthetic apparatus disappears during chromoplast formation, it would be expected that those plastid genes coding for proteins of the photosynthetic apparatus would cease to be expressed. Indeed Piechulla et al. (1985) reported decreases in the levels of ribosomal RNAs and of transcripts of *psa*A, *psa*B, and *psb*B; transcripts of *rbc*L, *psb*C, and *psb*D disappeared. No attempt was made in these studies to examine transcripts *not* associated with the photosynthetic

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Abbreviations: cDNA, complementary DNA; cpDNA, chloroplast DNA; cpRNA, chloroplast RNA; crRNA, chromoplast RNA; ORF, open reading frame; PSI, photosystem I; PSII, photosystem II; rRNA, ribosomal RNA; RuBPC, ribulose-1,5-bisphosphate carboxylase-oxygenase (E.C. 4.1.1.29); UORF, unidentified open reading frame

apparatus. In a study of chromoplast development in *Oenothera*, however, Bisanz-Seyer (1985) probed northern blots with fragments of plastid DNA and found some signals in crDNA that did not occur in cpDNA. Her data indicated the *possibility* of chromoplast-specific transcripts, but she points out that the data could not distinguish between transcripts from previously silent genes and differential processing of transcripts.

We chose the bell pepper, *Capsicum annuum* L., as our experimental material for several reasons: the genetics of fruit color and fruit development in pepper is well established (Hurtado-Hernandez and Smith 1985); it is one of few plants from which one can isolate intact chromoplasts from fully ripened fruit (Camara et al. 1982); and we had previously mapped the locations of 24 genes and conserved UORFs in this organism.

In searching for plastid-coded, chromoplast-specific genes, our strategy was to look for transcripts of the plastid genome that appear de novo or increase during chromoplast development. Specifically we have looked for regions of cpDNA that hybridize with crRNA but which do not hybridize or hybridize weakly with cpRNA. Our original tactics were to compare Southern blots of cpDNA probed with end-labelled plastid RNAs, but this method proved to be too insensitive. We subsequently obtained probes of sufficient sensitivity by constructing cDNAs complementary to crRNAs and cpRNAs using the random primer method of Taylor et al. (1976).

Materials and methods

Plant material. Green or red fruits of *Capsicum annuum* L., var. *Emerald Giant* were collected from the field and used within three days.

Extraction, digestion and blotting of DNA. The extraction of cpDNA and preparation of purified restriction fragments were described previously (Gounaris et al. 1986). Transfer of the restriction fragments to "Gene Screen Plus" membranes (New England Nuclear) was carried out as recommended by the supplier.

Extraction of RNA and construction of cDNA probes. 300 g of red or green pericarp was blended with 500 ml ice-cold buffer consisting of 0.35 M sucrose, 50 mM Tris-HCl, 10 mM Na_4 EDTA, 30 mM β-mercaptoethanol, and adjusted to pH 7.5. The tissue brei was filtered through four layers of Miracloth and a crude plastid pellet obtained by centrifugation at 1,500 g for 10 min. RNA was extracted from the plastids with guanidinium isothiocyanate-CsCl according to the method of Glisin et al. 1974.

cDNA probes labelled with [^{32}P]-dCTP and [^{32}P]-dATP to a specific activity of 5×10^6 to 10^7 cpm were constructed using the random primer method of Taylor et al. (1976). Herring sperm DNA was used for making the random primers.

Southern blots. Labelled cDNA at a concentration of 5×10^5 cpm/ml of hybridization buffer was hybridized with blotted cpDNA fragments overnight at 60 °C using the method of Church and Gilbert (1984).

Re-annealing chromoplast cDNA to cpRNA. In order to enrich chromoplast cDNA samples in chromoplast-specific cDNAs, the hybrids of crRNAs and chromoplast cDNAs were denatured in a boiling water bath for 10 min and immediately allowed to re-anneal to a ten-fold excess of unlabeled cpRNA similarly denatured. Re-annealing was carried out for 1 h at 65 °C using a final concentration of 0.3 μg of total nucleic acids per microliter. The samples were used immediately afterwards in Southern blot hybridization experiments.

Results

The objective of this work was to detect possible chromoplast-specific transcripts. Our tactics were to compare Southern blots of fragments of the plastid genome probed with cDNAs of crRNAs with those of cpRNAs. In order to detect possible chromoplast-specific transcripts amongst the predominantly chloroplast-specific transcripts, we sought to make the fragments small enough to contain no more than one gene. *Pvu*II is a convenient choice for the primary digest because it produces only 10 easily resolved fragments, four of which are doublets (Gournaris et al. 1986). The positions of these restriction fragments on the map of *Capsicum* cpDNA are shown in Fig. 1.

CpDNA of *C. annuum* was digested with *Pvu*II and the separated fragments cut from the gel. To decrease the size of the fragments further, each was digested with *Sac*I, *Eco*RI, and *Hae*II in combination. The subfragments produced from each *Pvu*II fragment were resolved on a 1.2% w/v agarose gel and found to range in size from 0.3 to 4.5 kbp. Most were between 2.5 and 1 kbp (Fig. 2a). Identical sets of such subfragments were blotted and probed with cDNAs prepared from cp- or crRNAs. The two probes produced very similar hybridization patterns (Fig. 2b, c). Each fragment produced hybridization signals with both probes. The strongest hybridization signals with both probes were obtained with subfragments of the 3.8- and 2.3-kbp fragments of *Pvu*II, where the rRNA genes are located. Both probes also gave strong signals with the 7.8-kbp *Pvu*II fragment, which is the locus for *psa*A and *psa*B. If the organization of *Capsicum* and tobacco cpDNAs were identical, the 7.8-kbp *Pvu*II fragment would also contain tRNA genes for serine, formyl-methionine and glycine, and UORFs 62 and 105 (Shinozaki et al. 1986).

The least intense signals were obtained with the subfragments of the 9.5-kbp fragment of *Pvu*II, which contains *atp*B and *atp*E. The corresponding fragment in tobacco (Shinozaki et al. 1986) contains genes for tRNAs for phenylalanine, lysine, threonine, serine,

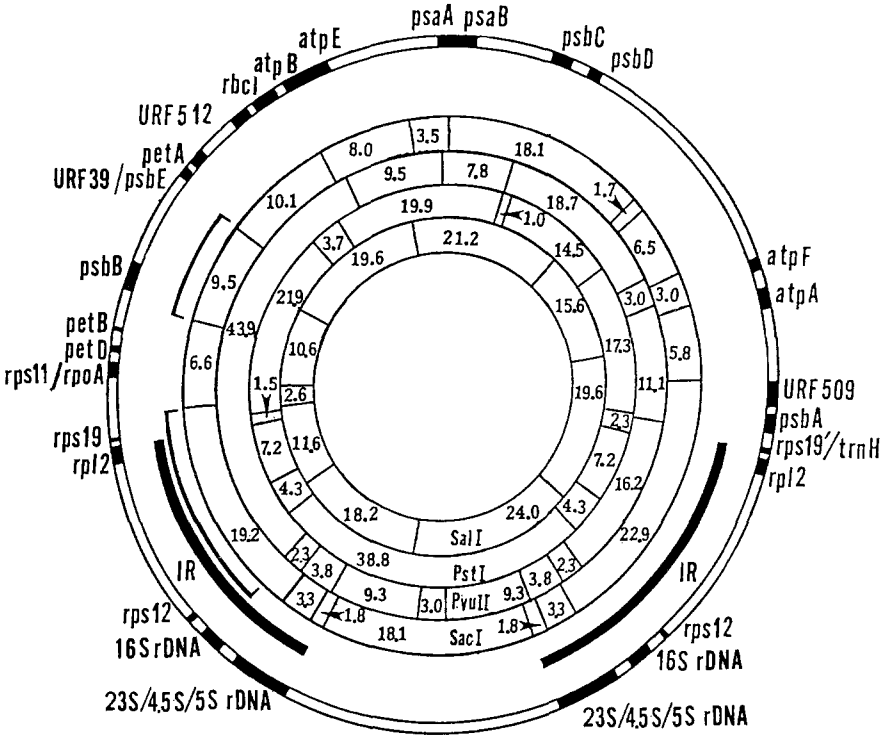

Fig. 1. Restriction and gene map of *Capsicum annuum* cpDNA (reproduced from Gounaris et al. 1986). *Brackets* indicate regions thought to be rich in UORFs

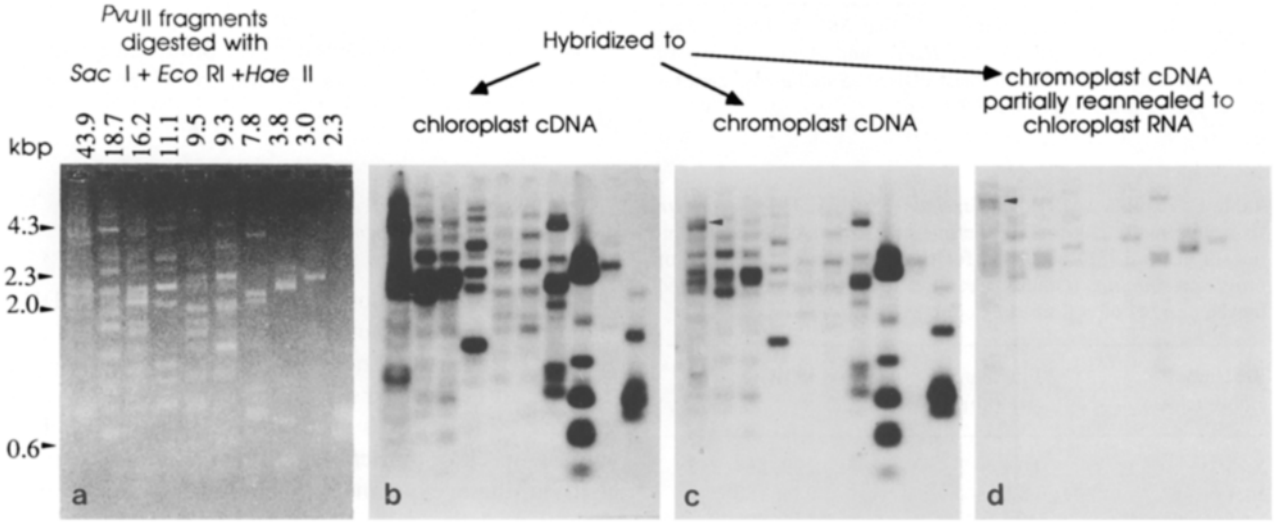

Fig. 2a–d. Detection of plastid transcripts in green and red fruits of *Capsicum annuum*. *Pvu*II fragments of *Capsicum* cpDNA were isolated and further digested with *Sac*I, *Eco*RI, and *Hae*II, and subjected to agarose-gel electrophoresis. *Lanes* are identified by the size of the original *Pvu*II fragment. Fragment sizes were estimated by comparison with *Hind*III fragments of λ. a DNA stained with ethidium bromide and visualized by fluorescence. b Southern *blot* of subfragments probed with cp-cDNA. c Southern *blot* of subfragments probed with cr-cDNA. d Southern *blot* of subfragments probed with cr-cDNA that had been reannealed with an excess of unlabelled cpRNA. *Small arrows* in (*c*) and (*d*) show the position of a 3.8-kbp fragment which shows an undiminished signal with reannealed cDNA

valine and methionine, ORF 284, ORF 158, and the putative gene *ndh*C. The contributions of these transcripts are obviously low.

We sought to increase the signal-to-noise ratio of chromoplast-specific sequences by removing sequences that are common to cr- and cpRNAs. To this end we allowed denatured cDNA-RNA hybrids from chromoplasts to re-anneal with a ten-fold excess of unlabeled cpRNA. Since cDNAs that correspond to RNAs that occur in both chromoplasts and chloroplasts will hybridize to their corresponding unlabeled cpRNA, the availability of the common sequences as probes will

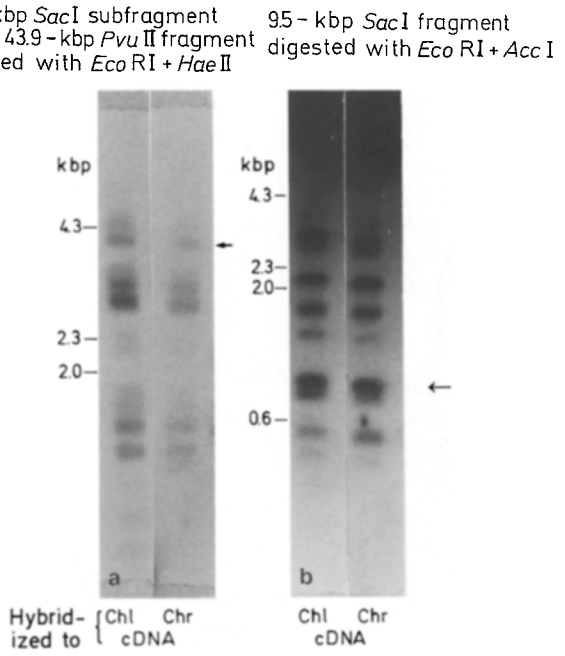

Fig. 3a, b. Further analysis of transcripts from regions of *Capsicum* plastid genome that showed possible chromoplast-specific signals. **a** 16.6-kbp *SacI-PvuII* fragment digested with *EcoRI* and *HaeII* and probed with cp- and cr-cDNAs. *Small arrow* denotes the expected locations of UORF 581. **b** 9.5-kbp *SacI* fragment digested with *EcoRI*, *HaeII*, and *AccI* and probed with chloroplast and chromoplast cDNAs. *Small arrow* denotes the expected locations of ORF 73

Table 1. Possible UORFs in *Capsicum* cpDNA. UORFs known to occur in the chloroplast genome of *Nicotiana tabacum* (Shinozaki et al. 1986) are listed for the homologous fragments of *Capsicum annuum* (Gounaris et al. 1986). UORFs are denoted by the number of codons they contain in *N. tabacum*

Restriction endonuclease	Fragment size (kbp)	UORFs
*Sac*I	10.1	229, 184, 512
*Sac*I	8.0	284, 158
*Sac*I	6.5	134, 80, 90
*Sac*I	3.5	77, 82
*Sac*I	1.7	151
*Pst*I	14.5	154, 151
*Pst*I	7.2	581, 1708
*Pst*I	3.7	512

be decreased. A Southern blot of cpDNA fragments hybridized to reannealed cr-cDNA is shown in Fig. 2d. Almost all of the bands showed decreased intensity compared to those probed with ordinary chromoplast cDNA, indicating that the sequences are present in both plastid types. The only exception was a 3.8-kbp subfragment of the 43.9-kbp fragment of *PvuII*, which showed equal intensity in both blots. Further examination of the 43.9-kbp *PvuII* fragment showed that the 3.8-kbp subfragment could be obtained from the 16.2-kbp *PvuII-SacI* region of cpDNA, which contains a large portion of the inverted repeat (marked by brackets in Fig. 1) and also from the 9.5-kbp *SacI* fragment after digestion with *EcoRI* and *HaeII*. Digestion of the 16.2-kbp *PvuII-SacI* subfragment of the 43.9-kbp *PvuII* fragment with *EcoRI* and *HaeII* in combination indeed produced the 3.8-kbp fragment. When this fragment was probed with cr- and cp-cDNAs separately, however, it hybridized equally with both probes (Fig. 3a). Similarly when the 9.5-kbp *SacI* fragment was digested with *EcoRI*, *HaeII*, and *AccI*, reducing it to subfragments ranging in size from 2.9 to 0.5 kbp, southern blots with chromoplast and chloroplast cDNAs were similar (Fig. 3b).

Tobacco cpDNA contains a number of UORFs (cf. Shinozaki et al. 1986). Since the locations of genes in the cpDNAs of pepper and tobacco are very similar (Gounaris et al. 1986), we examined eight restriction fragments of pepper cpDNA likely to contain these UORFs (Table 1). After further digestion of these fragments with *EcoRI*, *BamHI*, or a combination of both enzymes, the subfragments were resolved in 1.2% w/v agarose gel, blotted, and probed with cp- or cr-cDNAs. In each case the chromoplast probe produced a number of signals, but none were specific to the chromoplast (Fig. 4).

Discussion

Although Southern blots of *Capsicum annuum* cpDNA indicate that the chromoplasts of pepper fruits are rich in plastid transcripts, none of the hybridization signals appears to be peculiar to chromoplasts. Is this merely negative evidence, or is it proof of the absence of chromoplast-specific transcripts? In the strain of pepper used in these experiments, chromoplasts are formed from pre-existing chloroplasts. One might have expected that chloroplast transcripts would be degraded during the differentiation of the chloroplasts into chromoplasts, but many chloroplast transcripts are clearly retained in the mature chromoplast. The method employed relied on cutting cpDNA into pieces sufficiently small that residual chloroplast transcripts would not mask the appearance of a chromoplast-specific transcript. A 2-kbp fragment of cpDNA, however, could easily contain parts of two or more transcription units, so that the appearance of a chromoplast-specific transcript from such a fragment could be masked by the disappearance of a chloroplast-specific transcript. Given the presence of many chloroplast transcripts in crRNA, our method would have detected the appearance of an abundant

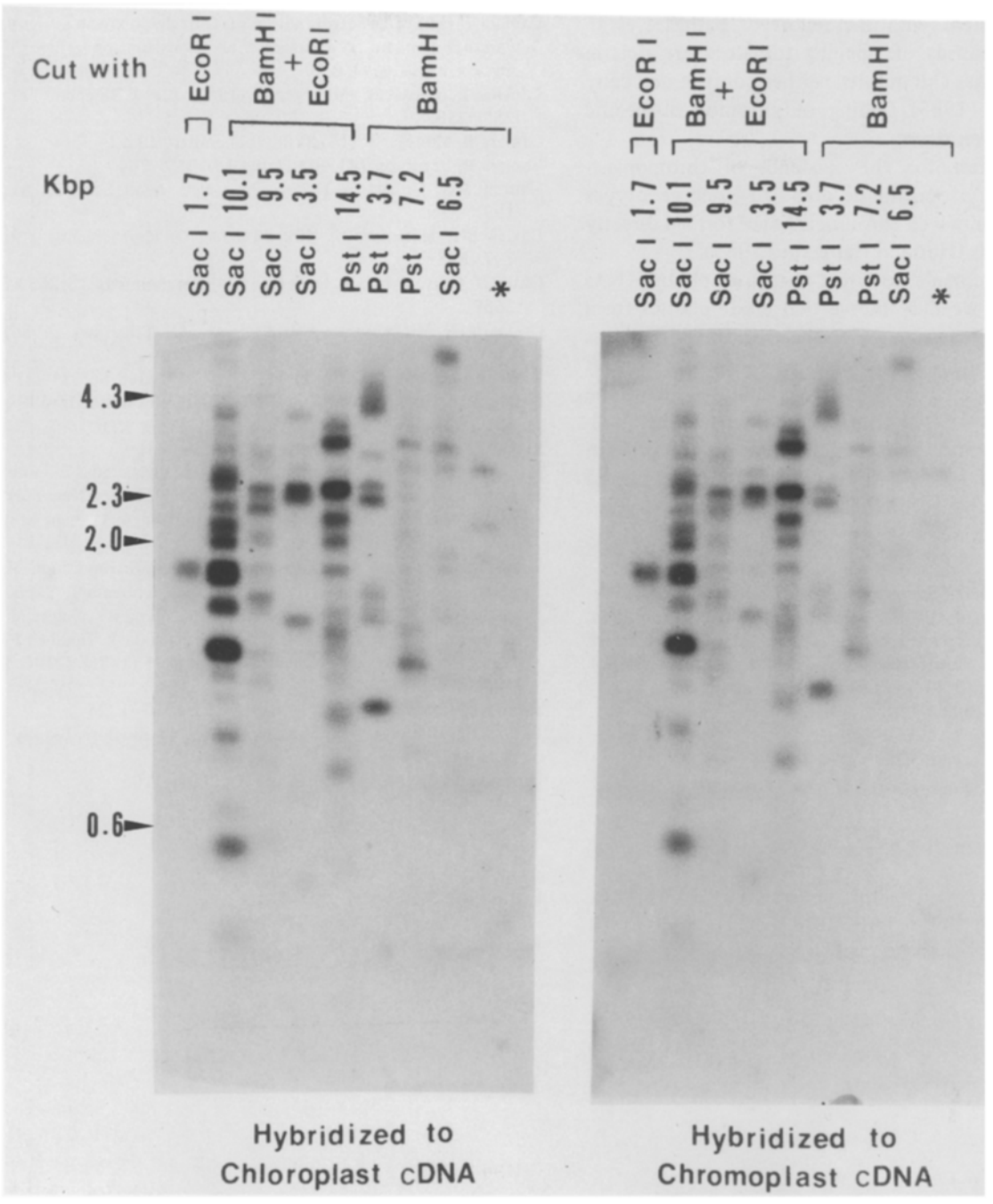

Fig. 4. Analysis of transcripts from regions of *Capsicum* cpDNA thought to contain UORFs. The fragments analyzed and their putative UORFs are described in Table 1. Sub-fragments produced with *Eco*RI and/or *Bam*HI were probed with cDNAs from cp- and cr-RNAs. The *asterisk* represents a 5.4-kbp *Pvu*I subfragment of the 6.5-kbp *Sac*I fragment

chromoplast-specific transcript, but could easily have missed a minor one.

The absence of detectable chromoplast-specific transcripts in regions of *Capsicum* cpDNA homologous with UORFs in the tobacco chloroplast genome must similarly be regarded as negative evidence. Taken together with what is known of the coding functions of cpDNA, our data make it unlikely but do not exclude the possibility that the plastid genome contributes to the development of chromoplasts.

Our data are consistent with the observations of Piechulla et al. (1985) that transcripts of several chloroplast genes are retained in chromoplasts of tomato. Our finding of strong signals to fragments containing rDNA

genes is consistent with the report of Bathgate et al. (1985) that plastids of ripening tomatoes are rich in ribosomal RNAs. Our results neither confirm nor deny Bisanz-Seyer's (1985) finding of chromoplast-specific transcripts in *Oenothera*.

A clearer test for the existence of chromoplast-specific transcripts might be obtained using genotypes of *C. annuum* in which chromoplasts are formed directly from proplastids (Hurtado-Hernandez 1985).

Although we made no effort to recover plastid RNAs quantitatively, we did observe that crude plastids from red fruits consistently yielded about three times as much RNA per unit fresh weight as plastids from green fruits. The increased yield of RNAs could be due to the relative softness of red fruits, but taken together with the high levels and variety of plastid transcripts shown by the Southern blots, it would appear that many plastid transcripts in the chromoplast are relatively stable or (less likely) continue to be synthesized.

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