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# Plastid gene expression during fruit ripening in tomato

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## Summary

A tomato chloroplast genome map has been constructed with the restriction enzymes *Hpa* I, *Pvu* II, and *Sal* I. Twelve plastid genes have been located on the tomato plastid genome (159 kb).

The expression of plastid genes during tomato fruit ripening has been studied. The levels of transcripts of various genes coding for proteins of the photosystem I (*psa*A), photosystem II (*psb*A, *psb*B, *psb*C, *psb*D) and the stroma (*rbc*L) decrease when plastids differentiate from chloroplasts to chromoplasts. The amount of plastid ribosomal RNA also decreases. Transcripts of the genes for the P700 reaction center protein (*psa*A), for the photosystem II-associated proteins (*psb*C, *psb*D) and for the large subunit of ribulose-1,5-bisphosphate carboxylase (*rbc*L) cannot be detected in chromoplasts. In contrast, a relatively high level of mRNA is present for the 32 kD protein ('herbicide-binding protein', *psb*A) in red fruit.

## Introduction

Tomato fruit ripening is a complex developmental process that has evolved for the production and dispersal of seeds. The fruit ripening process in *Lycopersicon esculentum* involves a series of distinct biochemical and structural changes. The chloroplasts in tomato undergo particularly drastic changes in ultrastructure and function during fruit maturation. This chloroplast/chromoplast transition is one example of tissue-specific differentiation of plastids found throughout plant development (56). The ultrastructural changes observed during chloroplast/chromoplast conversion in tomato fruit include the disappearance of the thylakoid membrane system and the degradation of chlorophyll (21, 22, 39, 51). Since the light-harvesting chlorophyll a/b binding proteins are unstable without chlorophyll, it is assumed that the photosynthetic apparatus breaks down during conversion (2, 3, 20). The early stage of chromoplast development is characterized by the appearance of plastoglobuli and increased carotenoid biosynthesis and/or accumulation (4, 47, 51). In the mature red fruit, the accumulated lycopene develops into a large crystal inside the chromoplast membranes. Currently, the precise sequence of events involved in controlling and executing these ultrastructural changes is unknown.

The ripening process in tomato fruit appears to be regulated by a number of exogenous and endogenous factors. Early experiments demonstrated that red light, via phytochrome, accelerates the synthesis and accumulation of lycopene in green tomatoes (31). In contrast, gibberellic acid inhibits lycopene synthesis and delays chlorophyll degradation (13). This inhibitory effect is reversible by either red light or the application of abscisic acid. Synthesis of abscisic acid is stimulated by red light prior to the accumulation of lycopene (32). The fruits at different ripening stages in avocado and tomato accumulate mRNAs for several polypeptides (e.g. cellulase or polygalacturonase) that are required for normal fruit ripening (6, 8, 16, 44, 45). It is unknown which regulatory mechanisms, if any, control the expression of nuclear and plastid genes en-

coding photosynthesis-specific polypeptides during the chloroplast/chromoplast transition. It is possible that several new polypeptides may be synthesized in, or imported into, the differentiating plastid (19); these include enzymes for the carotenoid biosynthetic pathway(s), most of which seem to be encoded in the tomato nuclear genome (48, 49).

We are interested in identifying the mechanism(s) that control the expression of nuclear and plastid genes during the chloroplast/chromoplast transition in ripening tomato fruit. In this report we have monitored the expression of photosynthesis-specific genes in the chloroplast of mature green fruit and during the development of the chromoplast. As expected, we have detected mRNA for all of the analyzed chloroplast genes in photosynthetically active green fruit, but not in the chromoplast of ripe tomato fruit. We find, however that the mRNA for the 32 kD protein of photosystem II (*psb*A) is still present at high levels in chromoplasts.

## Materials and methods

### *Plant material and tissue preparation*

Tomato plants (*L. esculentum,* cherry line, VFNT LA 1221) were grown under greenhouse conditions. Fruits were harvested at different developmental stages: premature green stage (fruit size 1.5–2.5 cm, approximately 30 days after pollination); yellow-orange intermediate stage (fruit size 3 cm, approximately 45 days after pollination); and fully ripe, red stage (fruit size 3 cm, approximately 50 days after pollination). Pericarp was isolated, immediately frozen in liquid nitrogen and stored at -70 °C.

### *Tomato chloroplast DNA restriction enzyme mapping and gene localization*

Chloroplasts were isolated from mature green leaves taken from young plants (6–8 weeks old). Plants were kept in the dark at 20 °C for 24 h prior to preparation in order to reduce the starch content. Leaf material was homogenized, in two batches of 60 gm, in 150 ml of 1× isolation buffer (1 × IB: 0.37 M sucrose, 10 mM Tris-HCl, pH 7.6, 50 mM EDTA, 0.1% BSA, 0.1% polyvinylpyrrolidone, 15 mM 2-mercaptoethanol, 1 mM spermine, 1 mM spermidine) using a Waring blendor with additional razor blades (30). The homogenate was filtered through four layers of miracloth. The filtrate was centrifuged at 1 000 rpm for 1 min (Sorvall SS34 rotor), to sediment starch, then for 10 min at 4 000 rpm, to collect the chloroplasts. The chloroplast pellet was resuspended in 1 × IB to a final volume of 20 ml. Chloroplasts were purified by floatation on sucrose, as described by Hallick *et al.* (18). The floated chloroplasts were recovered and washed with 1 × IB. After centrifugation, the chloroplast pellet was resuspended in a final volume of 10 ml in 1 × STED (0.37 M sucrose, 10 mM Tris-HCl, pH 7.6, 50 mM EDTA, 1 mM dithiothreitol), and the suspension was incubated with 0.5 ml pre-digested proteinase K (20 mg/ml) for 15 min at 60 °C. Chloroplasts were lysed by addition of one fifth volume of lysis buffer (5% sodium lauroylsarcosinate, 50 mM Tris-HCl, pH 8.0, 25 mM EDTA). The lysate was extracted with chloroform, phenol, and again with chloroform. Chloroplast DNA was precipitated with ice-cold ethanol for 5 min at -20 °C and collected by centrifugation (Sorvall SS34 rotor, 8 000 rpm, 5 min). The DNA pellet was resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) to a final concentration of 1 mg/ml.

A physical map of *L. esculentum* VFNT LA 1221 chloroplast DNA was generated using the restriction enzymes *Pvu* II, *Hpa* I, and *Sal* I. Restriction fragments were fractionated by agarose gel electrophoresis (0.5–0.7% agarose). The orientation of the fragments on the restriction enzyme map was determined using the Hutchison procedure for mapping DNA restriction fragments as described by Pero *et al.* (43).

Genes were localized to the tomato chloroplast DNA restriction-enzyme map by Southern hybridization (54) using gene-specific heterologous DNA probes from tobacco and spinach or synthetic oligonucleotides (Table 1).

### *Isolation of RNA*

Freshly harvested leaves (10 gm) were rinsed and frozen in liquid nitrogen. The frozen tissue was disrupted in a mortar, and the resulting tissue powder was resuspended in 50 ml buffer (0.35 M sorbitol, 50 mM Tris-HCl, pH 8.0, 25 mM EDTA, 15 mM

*Table 1.* Plasmids and synthetic oligonucleotide used for hybridizations.

| Plasmid | Restriction fragment | Source | Protein gene(s) | References |
|---|---|---|---|---|
| pSoc1080 | 8.2 kb, *Pst* I, | Spinach | *psa*A[a] | |
| pSoc910 | 20 kb, *Sal* I, | Chloroplast | *psa*A, *psb*C, *psb*D[a] | |
| pSoc800 | 12.3 kb, *Pst* I, | Chloroplast | *atp*B, *atp*E[a] | |
| pSocA | 1.2 kb, *Eco* RI-*Bam* HI, | Chloroplast | *pet*A[b] | |
| pSocB | 0.3 kb, *Xho* I, | Chloroplast | *pet*B[b] | |
| pSocD | 0.4 kd, *Bam* HI, | Chloroplast | *pet*D[b] | |
| pSD7 | 0.8 kb, *Xba* I-*Pst* I, | Chloroplast | *psb*A | (61) |
| Xho-8a | 3.0 kb, *Xho* I, | Chloroplast | *rrn* | (9) |
| pTB1 | 1.2 kb, *Bam* HI, | Tobacco | *rbc*L | (52) |
| pTB4 | 8 kb, *Bam* HI, | Chloroplast | *atp*A | (11) |
| pTB17 | 2.4 kb, *Bam* HI, | Chloroplast | *atp*B, *atp*E | (10) |
| pHA2 | 8.7 kb, *Hind* III, | Pea nuclear | *rDNA*[c] | |
| P680 | Nucleotides 1 – 50 of the spinach P680 protein coding region | | *psb*B | (37) |

[a] Plasmids pSoc1080, pSoc910 and pSoc800 contain the spinach chloroplast DNA restriction enzyme fragments *Pst* I#8, *Sal* I#2, and *Pst* I#5 in pBR322, respectively (E. Orozco, unpublished; see ref. 57).

[b] pSocA contains petA as a 1.2 kb *Eco* RI/*Bam* HI restriction enzyme fragment in pEMBL8. pSocB carries the internal 300 bp *Xho* I fragment #17 of the *pet*B in M13mp18. pSocD contains the internal 400 bp *Bam* HI fragment #29 in pEMBL8.

[c] The 8.7 kb *Hind* III fragment, including the pea nuclear ribosomal RNA genes, has been recloned from pHA1 into pBR322 to result in plasmid pHA2 (29).

2-mercaptoethanol, 2 mM dithiothreitol, 0.1% polyvinylpyrrolidone, 5 mM aurintricarboxylic acid). The homogenate was passed through four layers of miracloth. One-tenth volume of chloroplast lysis buffer (5% sodium lauroylsarcosinate, 50 mM Tris-HCl, pH 8.0, 25 mM EDTA) was added, and the homogenate was incubated for 15 min at room temperature. Protein was removed by two phenol:chloroform:isoamyl alcohol (25:25:1) extractions. One-tenth volume of 5 M ammonium acetate and 2 volumes of ethanol were added to the recovered aqueous phase to precipitate total nucleic acids. The nucleic acid pellet was resuspended in a small volume of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA), and 10 M lithium chloride was added to a final concentration of 2 M to selectively precipitate high-molecular-weight RNA (17). The RNA pellet was resuspended in diethylpyrocarbonate-treated $H_2O$ (38) and stored in aliquots at -20 °C.

The guanidinium/hot-phenol method (38) was used to isolate RNA from tomato fruits. Ten gm of frozen fruit pericarp was homogenized in 10 ml buffer (4 M guanidinium thiocyanate, 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 2% sodium lauroylsarcosinate, 1.4 mM 2-mercaptoethanol) for 15 sec in a Waring blendor. After addition of another 15 ml of the same buffer, the mixture was heated to 60 °C and subsequently passed through an 18-gauge syringe needle under high pressure. Thirty ml of preheated phenol, 3 ml of STE buffer (1 M sodium acetate, pH 5.2, 100 mM Tris-HCl, pH 7.4, 10 mM EDTA) and 50 ml chloroform:isoamyl alcohol (24:1) were added, followed by vigorous shaking of the emulsion for 15 min at 60 °C. After centrifugation (3 500 rpm, HB4 rotor, 10 min, 20 °C) the aqueous phase was extracted with phenol:chloroform:isoamyl alcohol (25:25:1) and once with chloroform. RNA was isolated from the aqueous phase as described above. The RNA pellet was resuspended in TE buffer containing 0.2% SDS, and pre-digested proteinase K (15 min, 37 °C) was added to a final concentration of 0.2 mg/ml. The suspension was incubated for 1.5 h at 37 °C. After heating to 60 °C, the suspension was extracted once with phenol and once with phenol:chloroform:isoamyl alcohol (25:25:1). Ethanol precipitated RNA was dried and resuspended in diethylpyrocarbonate-treated $H_2O$ and stored in aliquots at -20 °C. This method typically yielded 10–50 $\mu$g total RNA/g tissue.

### *Analysis and quantification of RNA*

Electrophoresis of RNA on formaldehyde-containing agarose gels was performed as described

by Maniatis *et al.* (38). The RNA was transferred from ethidium bromide stained or unstained formaldehyde gels to nitrocellulose filters. Filters were baked at 80 °C for 2 h under vacuum.

For quantification of specific RNA sequences, dilution series of LiCl-precipitated RNA in $H_2O$ (20 µl volume) were prepared immediately before binding to nitrocellulose. To remove secondary structure, the RNA was heated at 70 °C for 5–10 min, kept on ice and equilibrated to high salt conditions by adding 120 µl 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0). RNA dilutions were spotted onto nitrocellulose filters using a 'Dot-Blot' apparatus. The spotted RNA was washed twice with 200 µl 20 × SSC before the filter was baked 2 h at 80 °C under vacuum.

Nitrocellulose filters were prehybridized for at least 1 h at 65 °C in 500 ml 4 × SSC, 1 × Denhardt's solution (0.1 gm Ficoll, 0.1 gm polyvinylpyrrolidone, 0.1 gm BSA per 500 ml). Hybridizations with specific probes were carried out at 65 °C for 12–16 h in 6 × SSC, 1 × Denhardt's, and 0.5% SDS. The filters were washed at 45 °C, 55 °C or 65 °C with 1 L 2 × SSC (3 times 20 min) and 1 L 1 × SSC (3 times, 20 min) and exposed to X-ray films. When oligonucleotides (50 nucleotides) were used as hybridization probes, nitrocellulose filters were prehybridized in 6 × SSC, 20% formamide, 0.1% SDS, 0.1 mg/ml tRNA (yeast) at 42 °C for 1 h. The 5′ labeled oligonucleotides were added to the hybridization solution (see above) and hybridized at 42 °C for 12 h. Filters were washed twice for 5 min each at 20 °C in 2 × SSPE (300 mM NaCl, 23 mM $NaH_2PO_4 \times H_2O$, 2.2 mM EDTA, pH 7.4), 0.1% SDS, and once for 5 min in 0.1 × SSPE at 20 °C, 30 °C or 37 °C.

The autoradiograms obtained from northern blot hybridizations were scanned with a Joyce Loebl densitometer. Relative amounts of mRNA were determined by peak-area measurements.

### *Plasmid DNA isolation*

Plasmid DNAs were isolated by a modified cleared-lysate procedure (7, 27). The crude plasmid DNA fractions were treated with RNase A and proteinase K prior to centrifugation. Supercoiled plasmid DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide gradients.

## Results

### *Construction of the tomato chloroplast DNA map*

The construction of a chloroplast DNA restriction enzyme map for the tomato VFNT LA 1221 cherry line was accomplished for the restriction enzymes *Hpa* I, *Pvu* II, and *Sal* I. Tomato chloroplast DNA restriction fragments were ordered by use of data from the Hutchison procedure, as well as relevant data from hybridization with various heterologous gene probes (Fig. 1). The map generated by *Pvu* II and *Hpa* I digestion of the chloroplast DNA is similar to that found by Palmer and Zamir for the *L. esculentum* cultivar T6 (41). The sizes of comparable restriction fragments do not differ significantly from those reported (Table 2).

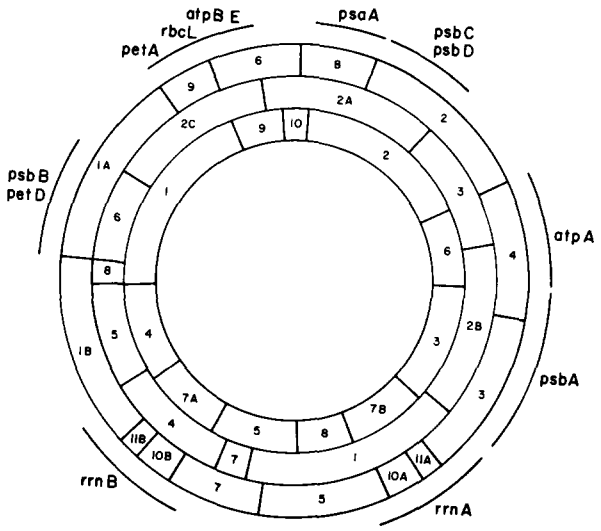


*Fig. 1.* Localization of protein encoding genes on the physical map of the tomato (*L. esculentum*, cherry line VFNT LA 1221) chloroplast genome. The genes were positioned by Southern hybridization. *atp*A, *atp*B, *atp*E - genes for the subunits of ATPase; *pas*A - gene for the P700 photosystem I reaction center protein; *psb*A - gene for the 32 kD thylakoid membrane protein; *psb*B - gene for the P680 photosystem II reaction center protein; *psb*C and *psb*D - genes for photosystem II associated proteins; *rbc*L - gene for the large subunit of ribulose-1,5-bisphosphate carboxylase; *pet*A - gene for cytochrome F; *pet*D - gene for the subunit 4 of the cytochrome b/f complex; *rrn* - genes for the large and small ribosomal RNAs.

Restriction sites for *Hpa* I (inner circle), *Pvu* II, and *Sal* I (outer circle) were determined on the tomato chloroplast genome. Restriction fragment sizes are given in Table 2.

*Table 2.* Restriction fragment sizes of chloroplast DNA from *L. esculentum* (VFNT LA 1221).

| *Pvu* II digest | Fragment size (kb) | *Hpa* I digest | Fragment size (kb) | *Sal* I digest | Fragment size (kb) |
|---|---|---|---|---|---|
| 1A | 22.1 | 1 | 31.0 | 1 | 28.1 |
| 1B | 21.2 | | | | |
| 2 | 19.8 | 2 | 27.0 | 2A | 23.0 |
| | | | | 2B | 22.2 |
| | | | | 2C | 21.6 |
| 3 | 18.3 | 3 | 18.0 | 3 | 16.7 |
| 4 | 15.3 | 4 | 15.4 | 4 | 15.3 |
| 5 | 14.6 | 5 | 13.5 | 5 | 13.1 |
| 6 | 10.4 | 6 | 11.9 | 6 | 11.9 |
| 7 | 10.2 | 7A, B | 11.2 | 7 | 4.3 |
| 8 | 8.5 | 8 | 8.6 | 8 | 2.9 |
| 9 | 6.0 | 9 | 7.6 | | |
| 10A, B | 4.1 | 10 | 3.5 | | |
| 11A, B | 2.3 | | | | |

The total size of the tomato chloroplast DNA genome was estimated to be 159 kb.

The location of the genes analyzed in this study, and their relative order on the tomato chloroplast DNA molecule, are identical to those reported for spinach and tobacco genes, although there appear to be small differences in the relative spacings between some genes (e.g., *pet*A on tomato chloroplast DNA is closer to the *rbc*L/*atp*B,E loci than it is in spinach). It should be noted that the endpoints for gene locations were not precisely mapped on the chloroplast DNA molecule but merely coincide with the endpoints of the fragments that hybridized to the gene probe.

*Changes in plastid mRNA during ripening*

RNA preparations from tomato leaves and different fruit ripening stages were analyzed on denaturing formaldehyde gels (Fig. 2A). Seven distinct bands (lane 1–4) were visible after ethidium bromide staining. Four bands were identified that correspond to the large and small cytoplasmic rRNAs (A and C) and to the large and small chloroplast rRNAs (B and D). It is possible that the remaining unidentified bands are specific ribosomal RNA fragments, which result from 'hidden' breaks in the secondary structure of the rRNA molecules (15). We cannot exclude, however that they also represent mitochondrial ribosomal RNAs that cross-hybridized with the DNA probe. Significant degradation of rRNA was not detected, although the background hybridization with green fruit RNA preparations (lane 2) was slightly higher than in the other RNA isolates.

The identification of specific mRNA sequences present during the chloroplast/chromoplast conversion in ripening tomato fruits was accomplished using northern blot analysis. RNA from different tissues and from different fruit ripening stages was standardized using spectrophotometric quantifications, intensity of ethidium bromide staining on agarose gels, and the relative levels of hybridization with heterologous cytoplasmic rDNA (pea; Fig. 2B and Fig. 5A). The normalized RNA amounts were used as the basis for detecting plastid gene expression by northern blot analysis.

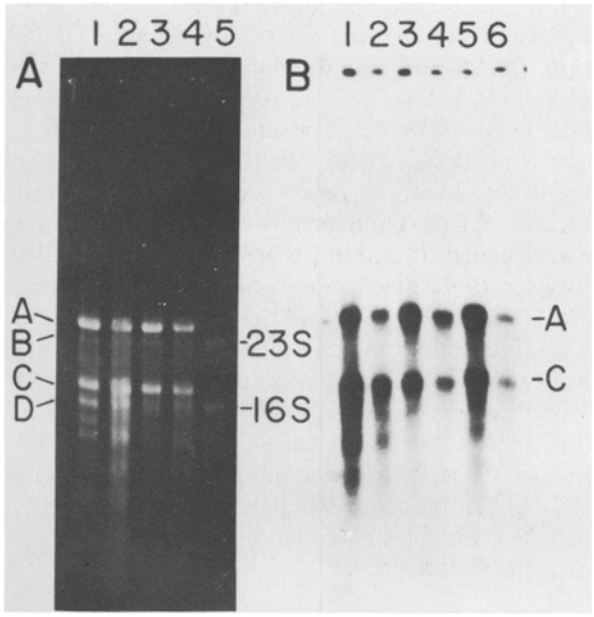


*Fig. 2.* Panel A. Separation of total RNA isolates from tomato on a denaturing formaldehyde gel after ethidium bromide staining: total RNA from leaves (lane 1), premature green fruits (lane 2), intermediate stage fruits (lane 3), and red fruits (lane 4). *E. coli* rRNA (lane 5) was separated into the large (23S) and small (16S) rRNA. The large and small cytoplasmic (A, C) and chloroplast (B, D) ribosomal RNAs are indicated.

Panel B. Hybridization of heterologous cytoplasmic rDNA (pea, pHA2) to total RNA from premature green fruits (lanes 1 and 2), intermediate stage fruits (lanes 3 and 4) and red fruits (lanes 5 and 6). Lanes 1, 3, and 5 were loaded with twice the amount of RNA as in lanes 2, 4, and 6 (5–10 $\mu$g total RNA per lane). The large (A) and small (C) cytoplasmic RNAs are indicated.

Northern blots of RNA isolated from tomato leaves and fruits were hybridized with heterologous chloroplast genes from spinach and tobacco. At present fifteen protein genes have been identified on the plastid genome map of spinach. Of these, we have used the following genes for our hybridization experiments: *psa*A, coding for the P700 protein of photosystem I, *psb*A, *psb*B, *psb*C, and psbD, coding for proteins associated with the photosystem II, *atp*A, *atp*B, *atp*E, coding for the subunits of the ATPase, and the gene for the large subunit of the ribulose-1,5-bisphosphate carboxylase (*rbc*L).

*Table 3.* Relative amounts (%) of plastid gene transcripts in RNA preparations from various ripening stages in tomato.

| Gene | Leaf | Green fruit | Intermediate fruit | Red fruit |
|---|---|---|---|---|
| Cytoplasmic rDNA* | 100 | 90.2 | 93.7 | 95.8 |
| Plastid | | | | |
| *rrn** | 100 | 44.1 | 37.2 | 16.2 |
| *rbc*L | 100 | 14.1 | 5.8 | n.d. |
| *psa*A | 100 | 20.8 | 4.4 | 0.7 |
| *psb*A | 100 | 69.9 | 35.1 | 20.4 |
| *psb*B | 100 | 63.5 | 13.9 | 4.5 |
| *psb*C or D | 100 | 37.5 | 3.2 | n.d. |
| *psb*D or C | 100 | 28.1 | 2.2 | n.d. |

n.d.: not detectable.

* Data combined from nothern blot scanning and scintillation counting of radioactivity on 'Dot-Blot' spots.

*Transcripts for proteins of photosystem I*

The gene coding for the P700 photosystem I reaction center protein (*psa*A) has been localized to the center of the large single copy DNA of the spinach plastid genome (57). The location of this gene was found to be similar in tomato chloroplast DNA (Fig. 1). The result from hybridization with the plasmid pSoc910, carrying the genes *psa*A, *psb*C and *psb*D from spinach, are shown in Fig. 3A. Three major transcripts were detected in total green leaf RNA. Based on hybridizations with the clone specific for *psa*A (pSoc1080), the largest transcript corresponds to the mRNA for the P700 photosystem I reaction center protein (Fig. 3A, band A). The length of the transcript was estimated to be 6 000 nucleotides. A *psa*A transcript of similar size has been reported in spinach (57). The amount of P700 transcript in green fruit is reduced to 27% of the RNA level found in leaves (Table 3). In red fruit the transcript decreases to almost undetectable levels. No hybridization signal was detected with the pBR322 vector DNA sequences in this or all subsequent experiments.

*Transcripts for proteins of photosystem II*

In most higher plant chloroplast DNAs, *psb*A and *psb*B are separated by approximately 70–80 kb, while *psb*C, and *psb*D are located close to *psa*A in the center of the large single copy region (58). Again, this gene organization was found to be similar on the tomato plastid genome (Fig. 1). The *psb*C gene in spinach codes for the chlorophyll a binding protein of photosystem II (44 kD protein), while the *psb*D gene encodes the so called 'D2'-protein (30–39.5 kD protein, 5). Both genes have been localized on a number of plastid genomes (1, 26, 47, 50). In spinach *psb*C and *psb*D overlap by 50 nucleotides and are thought to be cotranscribed (26). Hybridization of tomato leaf RNA with plasmid pSoc910 revealed three discrete transcripts. In Fig. 3A band B and band C correspond to the transcripts of *psb*C and *psb*D; each is approximately 3 000 nucleotides in length. The identities of the individual transcripts were not specifically matched to either cloned gene. In green fruit the level of transcripts for both genes is reduced to 28–37% of the amount found in leaf. During development to the intermediate stage fruit *psb*C and *psb*D mRNA levels decrease to 2–3%, while red fruit RNA contains no detectable amount of transcripts (Table 3).

Interestingly, a new high-molecular-weight band (approximately 7 000–8 000 nucleotides) cross-hybridized to pSoc910 in RNA preparations from fruit of all ripening stages (Fig. 3A). The level of this unidentified transcript decreases during the ripening process from 100% in green fruits to 45% in intermediate fruits and 9% in red fruits.

An oligonucleotide probe complementary to the first 50 nucleotides of the coding region of the P680 reaction center protein gene of photosystem II (40) was used to identify *psb*B transcripts in tomato RNAs isolated from leaves and fruits (Fig. 3B). The oligonucleotide hybridized to four distinct RNAs from chloroplast of leaves. Polycistronic transcription of *psb*B with the genes for cytochrome b6 (*pet*B) and subunit 4 (*pet*D) of the

cytochrome b/f complex has been proposed (23, 40). The final *psb*B transcript (Fig. 3B, band D) is approximately 2 000 nucleotides long. The expression of *psb*B is significantly reduced during the transition from chloroplast to chromoplast. However, it was possible to detect 5% of the leaf level of this RNA in chromoplasts from ripe fruit (Table 3).

Hybridization with an internal fragment (pSD7) of the gene for the 32 kD polypeptide of photosystem II (*psb*A) shows a single band of approximately 1 200 nucleotides (Fig. 3C). The size of this mRNA is in agreement with previously published sizes of *psb*A mRNA from mustard, tobacco and spinach (36, 55, 61). No precursor transcripts have been identified for any of the 32 kD protein transcripts from various higher plant species (42). Although quantitation of the mRNA indicates a decrease in the level of transcripts for the 32 kD protein during fruit ripening (Table 3), a surprisingly high level of this mRNA was detected in RNA from fully matured fruits. The mRNA is present at approximately 20% of the level found in leaf RNA. The relative amount of the 32 kD transcript in ripe fruit is significantly higher than the levels found for any of the transcripts encoding polypeptides of photosystem II, the P700 protein of photosystem I or the large subunit of ribulose-1,5-bisphosphate carboxylase.

*Transcripts for stromal proteins*

The gene for the large subunit of the ribulose-1,5-bisphosphate carboxylase (*rbc*L) is encoded in the large single copy region of spinach and tobacco chloroplast DNA. The organization of *rbc*L in tomato chloroplast DNA appears to be similar (Fig. 1). Hybridization of a *rbc*L specific probe with RNA from leaves and fruits of tomato revealed a single transcript of approximately 1 800 nucleotides (Fig. 4). Similar results have been obtained for the tobacco *rbc*L transcript (52). The

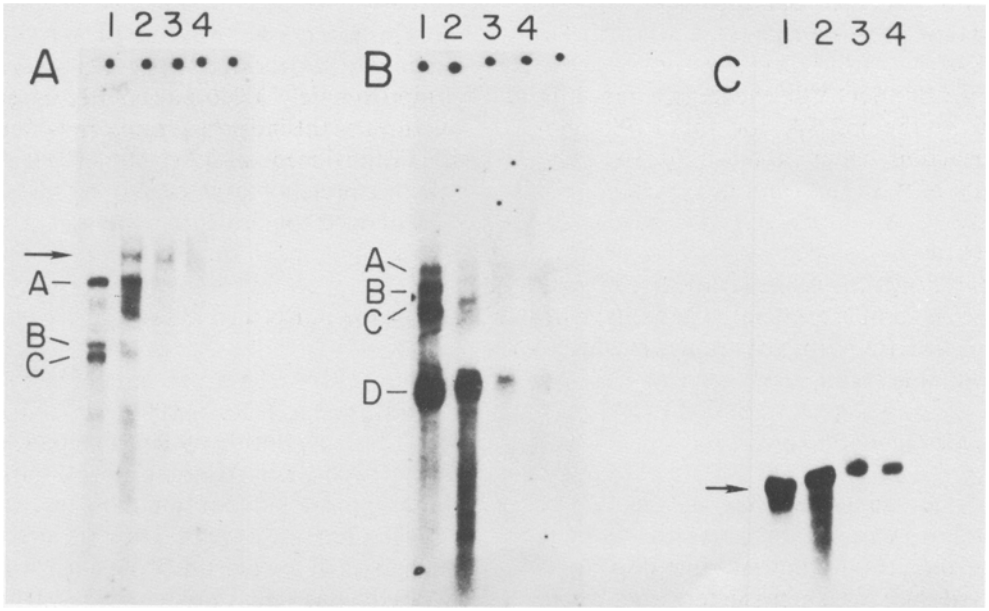


*Fig. 3.* Hybridization of heterologous plastid genes (Table 1) with total RNA isolated from leaves (lane 1), premature green fruits (lane 2), intermediate stage fruits (lane 3), and red fruits (lane 4). Autoradiograms were exposed for 5 days at -70 °C.

Panel A. Hybridization with nick-translated plasmid pSoc 910: Identification of the transcript of the P700 reaction center protein (*psa*A, A), the chlorophyll a/b binding protein (*psb*C), and the D2 protein (*psb*D) (B and C) of photosystem II. The arrow indicates a transcript occurring in fruit RNA preparations.

Panel B. Hybridization with the oligonucleotide complementary to the P680 reaction center protein (*psb*B). Bands indicated A, B, and C are cotranscripts of *psb*B, band D corresponds to the mRNA of the P680 protein of photosystem II.

Panel C. Hybridization with the plasmid pSD7, a 800 bp internal fragment of the 32 kD protein gene (*psb*A). The arrow indicates the transcript of *psb*A in tomato leaves and fruits.

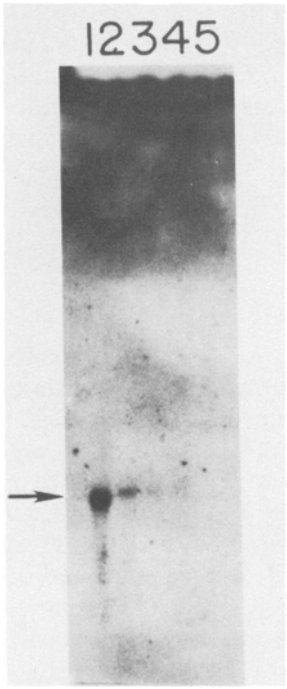


*Fig. 4.* Hybridization with nick-translated plasmid pTB1, carrying the gene for the large subunit of ribulose-1,5-bisphosphate from tobacco (exposure time: 5 days at -70 °C). The arrow indicates the transcript of *rbc*L in tomato leaf and fruit RNA preparations. Leaf RNA (lane 1), premature green fruit RNA (lane 2), intermediate stage fruit RNA (lane 3), red fruit RNA (lane 4), and *E. coli* rRNA (lane 5).

amount of *rbc*L mRNA decreases to 15% in green fruit and to 6% in intermediate stage fruits, when compared to leaf RNA. In red fruit *rbc*L mRNA was not detectable (Table 3).

*Transcripts for membrane proteins*

The genes for three subunits of the ATPase (*atp*A, B, E) are located in the large single copy region of the tomato chloroplast genome (Fig. 1). The organization of these genes in tomato is similar to positions described for other higher plants (12, 34, 53, 60, 62). Northern blot hybridizations using an atpB/E specific probe (pTB17) versus RNA from tomato leaves reveals three distinct transcripts of approximately 2 700, 2 000, and 800 nucleotides. In tobacco the *atp*B and *atp*E genes are cotranscribed as a 2 700 nucleotide polycistronic mRNA (53). Tomato fruit RNA isolates from different ripening stages contain high-molecular-weight RNA that hybridizes to the plasmid (data not shown). These mRNAs are longer than the cytoplasmic ribosomal RNA but do not resolve as discrete bands. High-molecular-weight bands were also observed when tomato RNAs from leaves and fruits were hybridized with a specific clone for the *apt*A subunit gene (pTB4). One major transcript of approximately 3 000 nucleotides was detected in leaf RNA. The tobacco *atp*A transcript has also been reported to be 3 000 nucleotides (12). Transcripts of varying sizes (at least 3 000 nucleotides long) hybridize to the atpA probe.

*Plastid ribosomal RNA*

Changes in the amount of ribosomal RNAs in plastids were determined by quantitations of 'Dot-blots' (Fig. 5) and of northern blots (not shown). The quantitated data from both methods were combined and are given in Table 3. The expected hybridization to rRNA from chromoplasts of red fruit as shown in Table 3 can be detected on 'Dot-blots' after extended exposure. The results are consistent with a decrease of plastid rRNA during the conversion of chloroplasts to chromoplasts in tomato fruits. The presence of relatively high levels of plastid ribosomal RNA in red fruit might indicate that protein biosynthesis may still occur in fully developed tomato chromoplasts.

## Discussion

The mRNA level of several photosynthetic plastid protein genes (*psa*A, *psb*A, B, C, D, and *rbc*L) decrease during the ripening process in tomato. In the late fruit ripening stages, the amount of transcripts are reduced to almost undetectable levels. This decrease of plastid mRNA levels is not due to changes of the plastid DNA/nuclear DNA ratio, which is constant throughout the fruit ripening process (data not shown). The levels of transcripts of these genes change at least 15–30 fold when chloroplasts differentiate into chromoplasts during tomato fruit ripening. We conclude that virtually no synthesis of the P700 reaction center protein (*psa*A), the chlorophyll a binding protein (*psb*C) or the 'D2'-protein (*psb*D) occurs in chromoplasts. It is unlikely, that in the absence of these photosyn-

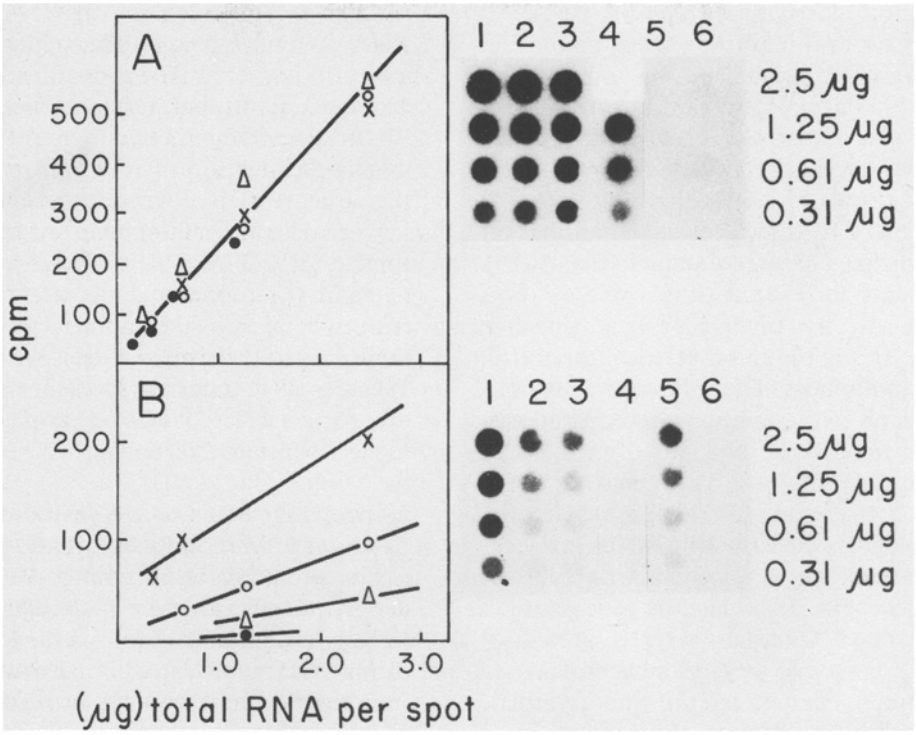


*Fig. 5.* 'Dot-Blot' analysis of RNA isolates from tomato leaves (1), premature green fruit (2), intermediate stage fruit (3), and red fruit (4); *E. coli* rRNA (5), and Hela total RNA (6). Panel A. Hybridization with the heterologous cytoplasmatic rDNA probe (pea, pHA2). The autoradiograms were exposed for 15 h at -70 °C. Panel B. Hybridization with the heterologous chloroplast *rrn* probe (spinach, *Xho*-8a).

The radioactive spots were excised from the nitrocellulose filter, the hybridization level was determined by scintillation counting, and the data were plotted. Leaf (×--×), premature green fruit (o--o), intermediate stage fruit (△--△), and red fruit (●--●).

thetic proteins photosynthesis still occurs in red tomato fruits. This conclusion is supported by our measurements of concentrations of the P700 reaction center protein (photosystem I) and the primary electron acceptor Q of the photosystem II in green and red fruits (data not shown). It should be mentioned that our results on plastid gene expression during fruit ripening were based on the assumption that the concentration of cytoplasmic rRNA does not vary significantly in diverse tissues and at different developmental stages. If cytoplasmic rRNA levels decrease during tomato fruit ripening (45), our calculated values of chloroplast mRNAs are progressively overestimated in the later stages of fruit maturation.

The key enzyme of the $CO_2$-fixation cycle (Calvin cycle), ribulose-1,5-bisphosphate carboxylase, is assembled from 8 large subunits (53–56 kD) and 8 small subunits (12–15 kD) (35). A single large subunit gene (*rbc*L) is located on the plastid genome, whereas a set of small subunit genes (*rbc*S) are nuclear encoded (24, 37, 59). In our experiments *rbc*L transcripts were not detected in chromoplasts, indicating that $CO_2$ fixation is probably reduced or even turned off in red fruits. This conclusion is supported by our measurements of ribulose-1,5-bisphosphate carboxylase activity in pericarp tissue from green fruit, intermediate stage fruit and red fruit (34.9%, 2.2%, 0.8%, respectively). In green fruit plastids the level of *rbc*L transcripts is reduced to 14%, compared to fully active chloroplasts in green leaves. The differences in (1) protein activities and (2) gene expression between chloroplasts from green fruits and leaves may be explained by their chemical composition. Chloroplasts from green fruit have a significantly higher content of starch reserves than do leaf chloroplasts. Therefore, green fruit plastids sometimes resemble

amyloplasts more closely than chloroplasts (51). At present it is not known how the expression of *rbc*L and *rbc*S genes is regulated during tomato fruit ripening. Preliminary results indicate that the expression of photosynthesis related nuclear and plastid encoded genes is not coordinately regulated.

The 32 kD thylakoid membrane protein (*psb*A) of photosystem II is known to be responsible for herbicide (atrazine, DCMU) binding in plants (59). Our experiments show decreasing levels of *psb*A transcripts during the process of fruit ripening, ranging from 70% in the chloroplasts of green fruit to 20% in chromoplasts. The relative level of *psb*A transcripts found in red fruits, however, is at least 20 fold higher than the mRNA level of most of the other genes described above. This could be due to an increased half-life time of this particular transcript or to a higher transcription rate for this gene. The 32 kD protein does not accumulate because of its rapid turnover (14, 25), while the gene products of the *psa*A, the P700 reaction center protein of photosystem I, appear to be very stable proteins. In order to maintain a certain level of photosynthetic activity, plastids in green tomato fruits have to synthesize stoichiometric amounts of the components for photosystems I, II and the electron transport chain. Due to the different stabilities of the 32 kD polypeptide and other components of photosystem II, it is possible that *psb*A is transcribed at a higher rate and/or its mRNA accumulates to increased levels. At present no data are available for the turnover rate of *psb*A mRNA in different tissues. Also, we do not know whether and to what extent the *psb*A mRNA is translated in red tomato fruit.

The ATPase protein complex is located in the thylakoid membrane. It is assembled from three nuclear and four chloroplast encoded gene products. The level of the *atp*A, B, and E transcripts were not determined in our experiments because no discrete transcripts were observed in RNA isolates from fruit tissue. It is very unlikely that this is due to degradation, because (1) identical RNA preparations were used for hybridizations with other plastid specific probes that always lead to discrete transcripts, and (2) the hybridizing RNAs have higher molecular weights than do the transcripts found in leaf RNA preparations. Interestingly, this phenomenon occurs only in RNA isolated from fruit tissue. When the plasmid pSoc910 was hybridized to a northern blot (Fig. 3A), an additional transcript was detected in fruit RNA but not in leaf RNA. Whether these results reflect fruit specific transcription, or are the consequence of a common experimental artifact, needs further investigations.

Ultrastructural and physiological alterations, the drastic degradation of thylakoid membranes, and the decrease of the chlorophyll content occur during chloroplast/chromoplast differentiation in tomato (21, 22, 28, 33, 39). These events are consistent with the molecular data presented here. During fruit ripening several components of the photosynthetic apparatus exhibited decreased mRNA levels. Whether these transcript levels in chromoplasts are due to a decreased rate of transcription or to a higher turnover rate remains unknown. Since we have found plastid rRNA to be present in red fruit tomato, it remains to be determined, whether a functional transcription/translation apparatus exists in chromoplasts. Experiments are currently underway to elucidate the molecular mechanism(s) for the regulation of photosynthetic-specific genes of the tomato nuclear and plastid genomes during fruit ripening and chromoplast differentiation.

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## References

1. Alt J, Morris J, Westhoff P, Herrmann RG: Nucleotide sequence of the clustered genes for the 44 kD chlorophyll a apoprotein and the '32 kD'-like protein of the photosystem II reaction center in the spinach plastid chromosome. Current Genetics 8:597-606, 1984.
2. Apel K, Kloppstech K: The effect of light on the biosynthesis of the light-harvesting chlorophyll a/b protein. Planta 150:426-430, 1980.

3. Bennet J: Biosynthesis of the light-harvesting chlorophyll a/b protein. Eur J Biochem 118:61–70, 1981.
4. Ben-Shaul Y, Naftali Y: The development and ultrastructure of lycopene bodies in chromoplasts of *Lycopersicon esculentum*. Protoplasma 67:333–344, 1969.
5. Chua N-H, Gilham NW: The sites of synthesis of the principal thylakoid membrane polypeptide in *Chlamydomonas reinhardii*. J Cell Biol 77:441–452, 1977.
6. Christofferson RE, Tucker ML, Laties GG: Cellulase gene expression in ripening avocado fruit: The accumulation of cellulase mRNA and protein as demonstrated by cDNA hybridizations and immunodetection. Plant Mol Biol 3:385–391, 1984.
7. Clewell DB, Helinski DR: Supercoiled circular DNA-protein complex in *Escherichia coli:* Purification and induced conversion to an open circular DNA form. Proc Natl Acad Sci USA 62:1159–1166, 1969.
8. Crookes PR, Grierson D: Ultrastructure of tomato fruit ripening and the role of polygalacturonase isoenzymes in the cell wall degradation. Plant Physiol 72:1088–1093, 1983.
9. Crouse EJ, Schmitt JM, Bohnert H-J, Gordon K, Driesel AJ, Herrmann RG: Intramolecular compositional heterogeneity of *Spinacia* and *Euglena* chloroplast DNAs. With an appendix: Bohnert H-J, Gordon K, Driesel AJ, Crouse EJ, Herrmann RG: Mapping of genes on the restriction endonuclease site map of *Spinacia oleracea* chloroplast DNA. In: Akoyunoglou G, Argyroudi-Akoyunoglou JH (eds) Chloroplast development. Elsevier, Amsterdam, 1978, pp 565–572.
10. Deno H, Shinozaki K, Sugiura M: Nucleotide sequence of tobacco chloroplast gene for the alpha subunit of proton-translocating ATPase. Nucl Acid Res 11:2185–2191, 1983.
11. Deno H, Kato A, Shinozaki K, Sugiura M: Nucleotide sequences of tobacco chloroplast genes for elongator $tRNA^{Met}$ and $tRNA^{Val}$(UAC): the $tRNA^{Val}$(UAC) gene contains a long intron. Nucl Acid Res 10:7511–7520, 1982.
12. Deno H, Shinozaki K, Sugiura M: Structure and transcription pattern of a tobacco chloroplast gene coding for subunit III of proton-translocating ATPase. Gene 32:195–201, 1984.
13. Dostal HC, Leopold AC: Gibberellin delays ripening of tomatoes. Science 158:1579–1580, 1967.
14. Ellis RJ: Chloroplast proteins: synthesis, transport, and assembly. Ann Rev Plant Physiol 32:111–137, 1981.
15. Grierson D: Electrophoresis of RNA. In: Rickwood D, Hames BD, Grierson D: Electrophoresis of RNA. In: Rickwood D, Hames BD (eds) Gel Electrophoresis of Nucleic Acids: A Practical Approach. IRL press, Oxford, Washington DC, 1982, pp 1–38.
16. Grierson D, Slater A, Speirs J, Tucker GA: The appearance of polygalacturonase mRNA in tomatoes: one of a series of changes in gene expression during development and ripening. Planta 163:263–271, 1985.
17. Hall TC: Plant messenger RNA. In: Hall TC, Davies JW (eds) Nucleic Acids in Plants. CRC Press, Boca Roton, Florida, 1979, pp 217–225.
18. Hallick RB, Richards OC, Gray PW: Isolation of intact, superhelical chloroplast DNA from *Euglena gracilis*. In: Edelman M, Hallick RB, Chua N-H (eds) Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, Amsterdam, New York, Oxford, 1982, pp 281–293.
19. Hausmann P, Sitte P: Comparison of the polypeptide complement of different plastid types and mitochondria of *Narcissus pseudonarcissus*. Z Naturf 39c:758–766, 1984.
20. Harpster MH, Mayfield SP, Taylor WC: Effects of pigment-deficient mutants on the accumulation of photosynthetic proteins in maize. Plant Mol Biol 3:59–71, 1984.
21. Harris WM, Spurr AR: Chromoplasts of tomato fruits: I. Ultrastructure of low-pigment and high-beta mutants: carotene analysis. Amer J Bot 56:369–379, 1969.
22. Harris WM, Spurr AR: Chromoplasts of tomato fruits: II. The red tomato. Amer J Bot 56:380–389, 1969.
23. Heinemeyer W, Alt J, Herrmann RG: Nucleotide sequence of the clustered genes for the apocytochrome *b*6 and subunit 4 of the cytochrome *b/f* complex in the spinach plastid chromosome. Current Genetics 8:543–549, 1984.
24. Highfield PE, Ellis RJ: Synthesis and transport of the small subunit of chloroplast ribulose bisphosphate carboxylase. Nature 271:420–424, 1978.
25. Hoffmann-Falk H, Mattoo AK, Marder JB, Edelmann M, Ellis RJ: General occurrence and structural similarity of the rapidly synthesized, 32 000-dalton protein of the chloroplast membrane. J Biol Chem 257:4583–4587, 1982.
26. Holschuh K, Bottomley W, Whitfeld PR: Structure of the spinach chloroplast genes for the D2 and 44 kD reaction center proteins of photosystem II and for $tRNA^{Ser}$(UGA). Nucl Acid Res 12:8819–8834, 1984.
27. Humphreys GO, Willshaw GA, Anderson ES: A simple method for the preparation of large quantities of pure plasmid DNA. Biochim Biophys Acta 383:457–463, 1975.
28. Iwatsuki N, Moriyama R, Asahi T: Isolation and properties of intact chromoplasts from tomato fruits. Plant and Cell Physiol 25:763–768, 1984.
29. Jorgensen RA, Cuellar RE, Thompson WF: Modes and tempos in the evolution of nuclear-encoded ribosomal RNA genes in legumes. Carnegie Institution of Washington Year Book 81:98–101, 1981–1982.
30. Kannangasa CG, Gough SP, Hansen B, Rasmussen JN, Simpson DJ: A homogenizer with replaceable razor blades for bulk isolation of active barley plastids. Carlsberg Res Commun 42:431–439, 1977.
31. Khudairi AK, Arboleda OP: Phytochrome-mediated carotenoid biosynthesis and its influence by plant hormones. Plant Plantarum 24:18–22, 1971.
32. Khudairi AK, Juglekar R: Phytochrome-mediated abscisic acid synthesis in tomatoes. Plant Physiol 47:11, 1971.
33. Khudairi AK: The ripening of tomatoes. American Scientists 60:696–707, 1972.
34. Krebbers ET, Larrinua IM, McIntosh L, Bogorad L: The maize chloroplast genes for the beta and epsilon subunits of the photosynthetic coupling factor $CF_1$ are fused. Nucl Acid Res 10:4985–5002, 1982.
35. Kung SD, Sakano K, Wildman SG: Multiple peptide composition of the large and small subunits of *Nicotiana tabacum* fraction I protein ascertained by fingerprinting and electrofocusing. Biochim Biophys Acta 365:138–147, 1974.
36. Link G, Langridge U: Structure of the chloroplast gene for the precursor of the $M_r$ 32 000 photosystem II protein from mustard (*Sinapis alba* I). Nucl Acid Res 12:945–958, 1984.
37. Lorimer GH: The carboxylation and oxygenation of

ribulose-1,5-bisphosphate: The primary events in photosynthesis and photorespiration. Ann Rev Plant Physiol 32:349-389, 1981.
38. Maniatis T, Fritsch EF, Sambrook J: Molecular cloning, a laboratory manual. Cold Spring Harbor, 1982.
39. Mohr WP, Stein M: Fine structure of fruit development in tomato. Can J Plant Sci 49:549-533, 1969.
40. Morris J, Herrmann RG: Nucleotide sequence of the gene for the P680 chlorophyll a apoprotein of the photosystem II reaction center from spinach. Nucl Acid Res 12:2837-2849, 1984.
41. Palmer JD, Zamir D: Chloroplast DNA evolution and phylogenetic relationships in *Lycopersicon*. Proc Natl Acad Sci USA 79:5006-5010, 1982.
42. Palmer JD, Edwards H, Jorgensen RA, Thompson WF: Novel evolutionary variation in transcription and location of two chloroplast genes. Nucl Acid Res 10:6819-6832, 1982.
43. Pero J, Hannett NM, Talkington C: Restriction cleavage map of SP01 DNA: General location of early, middle, and late genes. J Virol 31:156-171, 1979.
44. Rattanapanone N, Grierson D, Stein M: Ribonucleic acid metabolism during the development and ripening of tomato fruits. Phytochemistry 16:629-633, 1977.
45. Rattanapanone N, Speirs J, Grierson D: Evidence for changes in messenger RNA content related to tomato fruit ripening. Phytochemistry 17:1485-1486, 1978.
46. Rasmussen OF, Bookjans G, Stummann BH, Henningsen KW: Localization and nucleotide sequence of the gene for the membrane polypeptide D2 from pea chloroplast DNA. Plant Mol Biol 3:191-199, 1984.
47. Raymundo LC, Chichester CO, Simpson KL: Light-dependent carotenoid synthesis in tomato fruit. J Agric Food Chem 24:59-64, 1976.
48. Rick CM: Cytogenetics of the tomato. Adv Genet 8:267-382, 1956.
49. Rick CM: The tomato. In: King RC (ed) Handbook of Genetics, Vol 2. Plenum Press, New York, 1975, pp 247-280.
50. Rochaix JD, Dron M, Rahire M, Malnoe P: Sequence homology between the 32 K dalton and the D2 chloroplast membrane polypeptide of *Chlamydomonas reinhardii*. Plant Mol Biol 3:363-370, 1984.
51. Rosso SW: The ultrastructure of chromoplast development in red tomato. J Ultrastruc Res 25:307-322, 1968.
52. Shinozaki K, Sugiura M: The nucleotide sequence of the tobacco chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase. Gene 20:91-102, 1982.
53. Shinozaki K, Deno H, Kato A, Sugiura M: Overlap and cotranscription of the genes for the beta and epsilon subunits of tobacco chloroplast ATPase. Gene 24:147-155, 1983.
54. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503-517, 1975.
55. Sugita M, Sugiura M: Nucleotide sequence and transcription of the gene for the 32 000 dalton thylakoid membrane protein from *Nicotiana tabacum*. Mol Gen Genet 195:308-313, 1984.
56. Thomson WW, Whatley JM: Development of nongreen plastids. Ann Rev Plant Physiol 31:375-394, 1980.
57. Westhoff P, Alt J, Nelson N, Bottomley W, Buenemann H, Herrmann RG: Genes and transcripts for the P700 chlorophyll a apoprotein and subunit 2 of the photosystem I reaction center complex from spinach thylakoid membranes. Plant Mol Biol 2:95-107, 1983.
58. Westhoff P, Alt J, Herrmann RG: Localization of the genes for the two chlorophyll a conjugated polypeptides (mol. wt. 51 and 44 kD) of the photosystem II reaction center on the spinach plastid chromosome. EMBO J 2: 2229-2237, 1983.
59. Whitfeld PR, Bottomley W: Organization and structure of chloroplast genes. Ann Rev Plant Physiol 34:279-310, 1983.
60. Zurawski G, Bottomley W, Whitfeld PR: Structures of the genes for the beta and epsilon subunits of spinach chloroplast ATPase indicate a dicistronic mRNA and an overlapping translation stop/start signal. Proc Natl Acad Sci USA 79:6260-6264, 1982.
61. Zurawski G, Bohnert HJ, Whitfeld PR, Bottomley W: Nucleotide sequence of the gene for the $M_r$ 32000 thylakoid membrane protein from *Spinacia oleracea* and *Nicotiana debneyi* predicts a totally conserved primary translation product of $M_r$ 38950. Proc Natl Acad Sci USA 79:7699-7703, 1982.
62. Zurawski G, Clegg MT: The barley chloroplast DNA *atp* beta, epsilon, *trn*M2, and *trn*V1 loci. Nucl Acid Res 12:2549-2559, 1984.

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