Plant Physiol. (1988) 87, 239–243
0032-0889/88/87/0239/05/$01.00/0

# Abundance of the Major Chloroplast Polypeptides during Development and Ripening of Tomato Fruits

## AN IMMUNOLOGICAL STUDY

Received for publication August 17, 1987 and in revised form December 1, 1987

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### ABSTRACT

**During maturation and ripening of tomato (*Lycopersicon esculentum*, cv Tamar) fruits, there are differential changes in the steady state levels of chloroplast proteins. Western blot analysis indicated that with the exception of the core polypeptide of photosystem I (PSI) (subunit I) the whole complex disappears during the transition of chloroplast to chromoplast. The amounts of the core polypeptide of photosystem II (PSII) (43 kilodaltons) and the light harvesting chlorophyll protein complex increase during maturation and decrease thereafter. In contrast, the 33 kilodalton subunit of PSII is found at the highest levels from the early recorded stages and decreases gradually until late stages of ripening. The level of cytochrome *f* decreases slowly during the maturation and ripening process, whereas the Rieske protein of the same complex disappears at a faster rate. There are also differential changes in the subunits of the chloroplast coupling factor·ATPase complex; $\alpha$ and $\beta$ subunits increase during maturation, whereas the level of the $\gamma$ subunit is already maximal at the earliest recorded stage of development and depleted thereafter. The two subunits of the ribulose-1,5 bisphosphate carboxylase increase in abundance during chloroplast maturation and gradually disappear after the transition from chloroplast to chromoplast. However, there are substantial differences in the rates of increase and disappearance of the large and small subunits of this enzyme. This imbalance is attributed to different regulation of nuclear and chloroplast gene expression. In addition, the steady state levels of chloroplastic superoxide dismutase and phosphoenolpyruvate carboxylase have been followed. Both enzymes reach their maxima at the final stages of ripening. This increase coincides with the climacteric rise of $CO_2$ release.**

Mature green tomato fruits are capable of assimilating $CO_2$ photosynthetically, mainly in the external pericarp where the highest photosynthetic activity has been recorded (2, 11, 17, 34). The photosynthetic assimilation of these fruits indicates the presence of functional components of the photosynthetic apparatus and suggests a possible significance of this process for fruits. It has been suggested that the $CO_2$ evolved during respiration in fruits is refixed and recycled through the process of photosynthesis (2, 33, 34). Green tomato fruits contain chloroplasts that show the typical ultrastructural features of leaf chloroplasts, namely, grana, which contain the components involved in the light reactions, and the stroma where the Calvin cycle enzymes are localized (1). Though there is considerable information regarding the composition, function, and structural organization of the main polypeptide complexes in leaf thylakoids, little is known about the structure, stoichiometry, and organization of tomato fruit chloroplasts. During maturation and fruit ripening, the chloroplasts undergo gradual ultrastructural changes including membrane disintegration, Chl breakdown, and starch disappearance (1, 8, 16). The content of carotenoids increases and lycopene is synthesized in the cytoplasm and accumulated within inner membranes during the transformation from chloroplasts to chromoplasts (4, 7, 13, 14). This transformation takes place at the same developmental stage that other ripening processes such as cell wall degradation and softening and accumulation of sugars and organic acids are occurring (12). The transformation of chloroplasts, as well as the whole ripening process, is not a deteriorative and aging process *per se*. Rather, it is characterized by a series of events which include *de novo* synthesis and activation of specific enzymes which are significant for the normal ripening process. Active protein synthesis during ripening has been demonstrated by Spires *et al.* (30) and includes the synthesis of invertase (12), polygalacturonase (6, 12, 28), pectin methyl esterase (12, 28), and other enzymes. Comparison of protein profiles of chloroplasts and chromoplasts following separation on SDS-PAGE revealed many differences both quantitatively and qualitatively (12, 30). During the transition from chloroplast to chromoplast, there is a decrease in many polypeptides as well as a substantial increase in specific polypeptides (6, 12, 24). Labeling of proteins *in vivo* with [$^{35}$S]methionine has demonstrated differences in the rate at which specific proteins are synthesized (1). Little is known regarding the identity of the chloroplastic polypeptides that undergo changes during the development of fruits and the transition of chloroplasts to chromoplasts. The present study has focused on molecular events accompanying development and the transformation of chloroplasts to chromoplasts. In particular, we have used immunological techniques to follow selective changes in several of the principal polypeptide components of the photosynthetic apparatus.

## MATERIALS AND METHODS

**Preparation of Protein Samples.** Tomato (*Lycopersicon esculentum*, cv Tamar) fruits at various stages of development were harvested in the field. Eight developmental stages were classified according to fruit color and size: (a) green, 10 to 15 mm (diameter); (b) green, 15 to 30 mm; (c) green, fully expanded (mature); (d) green yellowish; (e) yellow; (f) orange; (g) orange red; and (h) dark red and soft. Sampling was confined to the outer layer (2 mm) of the pericarp since it contains a high density of chloroplasts and the highest photosynthetic activity of all tissues in the fruit (11, 17, 34). Approximately 300 mg of the tissue were homogenized at 4°C in 7 ml of 100 mM Tris (pH 8.0). The homogenate was filtered through four layers of cheesecloth, and 0.15 ml of a solution containing 1.85 M NaOH and 7.4% 2-mercaptoethanol were added for each ml of filtrate. After 10 min at 4°C, 150 $\mu$l of 50% TCA were added for each ml of solution, and the solution was held for an additional 10 min at

4°C before centrifuging at 17,000 rpm (Beckman-Rotor 20) for 10 min at 4°C. The pellet was suspended in 10 ml of 80% acetone and recentrifuged. The acetone was removed and the pellet dried with nitrogen and then solubilized in 2% SDS. All samples were stored at −20°C before electrophoretic separation.

**Electrophoresis and Immunoblotting.** Since the percentage of total protein per fresh weight was constant throughout the stages of fruit development (data not shown), the samples were calculated on fresh weight basis. Protein samples were loaded and electrophoresed on 12.5% SDS-polyacrylamide gels using a Laemmli discontinuous system. The electrotransfer of proteins was followed by immunodetection with specific antibodies as previously described (3, 9, 31). Quantitation of steady state levels of specific polypeptides was carried out with Beckman DU-8 spectrophotometer. Six biological replicates were carried out, and representative experiments are depicted here.

**Isolation of the PSI Complex and Preparation of Antibodies.** The PSI complex was isolated from leaves of Swiss chard following the method of Nechushtai *et al.* (19, 20). The main subunits were separated on SDS-PAGE and were injected into rabbits as previously described (20). Antibodies against subunits of the following chloroplast polypeptides were prepared in our laboratory: RuBPcase[1] (35), Cyt $b_6/f$ as described previously (15), ATPase (20–22), and the PSII complex as described previously (5, 18). The specificity of the antibodies was confirmed by comparison of their reactivity in crude tomato extracts with that of antibodies kindly donated by Dr. N. Nelson (18–21). Antibodies against the chloroplast SOD and PEPcase were prepared as previously described (29, 32).

## RESULTS

Figure 1 depicts the changes in the amounts of the three subunits of the $CF_1$ complex during development and ripening of tomato fruits. The $\alpha$- and $\beta$-subunits increased during the early stages of maturation, reached their highest levels at stage c (mature green), and then decreased as ripening and senescence progressed (Table I). In contrast, the amount of the $\gamma$-subunit was at its highest level during the earliest recorded stage of development (a) and decreased gradually thereafter. A similar trend of disappearance of the three $CF_1$ subunits has been demonstrated. During the final stages of senescence, detectable amounts of the polypeptides were still present (Fig. 1) indicating incomplete destruction of this complex.

Changes in the five subunits of the PSI complex are shown in Figure 2. The 'core' of the PSI (subunit I, 66–70 kD) was stable throughout development and ripening, but amounts of subunits II, III, VI and VII increased until stage c (mature green) and then decreased during the remainder of the ripening process (Fig. 2). With the exception of subunit I, the complex of PSI disappeared during the transition of chloroplasts to chromoplast (stages d and e).

Data on the levels of Cyt $b_6/f$ are included in Figure 3 (1, 2). Cyt*f* (34kD) was relatively stable throughout the maturation and ripening process and disappeared only at the terminal stages of senescence. In contrast, the Rieske protein (subunit III of this complex) disappeared, and at stage f the polypeptide was not detected.

The PSII is represented in Figure 3 by three subunits: the 43 kD core polypeptide, the 33 kD polypeptide, and the LHCP complex. The 43 kD polypeptides and the LHCP complex are both associated with Chl; both subunits increased significantly (1.5- to 2.5-fold) during maturation of the tomato fruit (stage a to c), but both were degraded during subsequent ripening and senescence. In contrast, the 33 kD subunit was at its highest level from the earliest stages of fruit development (a), and its level remained relatively constant until stage e. All three peptides of the PSII complex completely disappeared during the ripening stage.

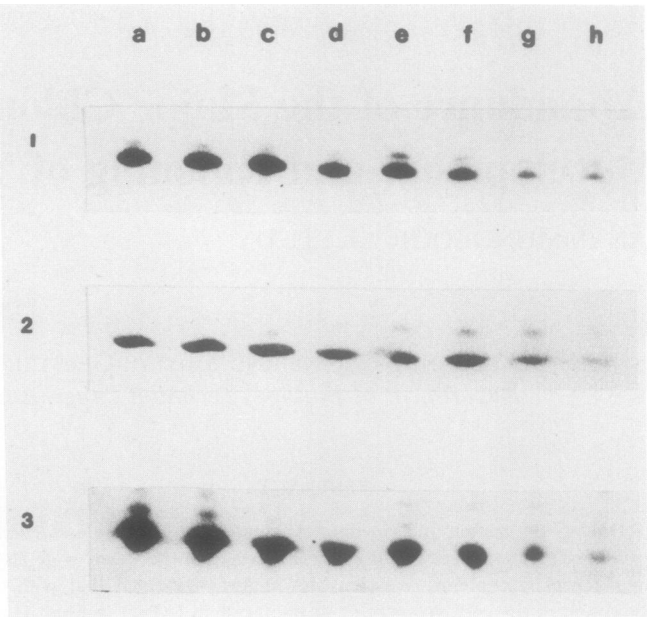


FIG. 1. Changes in the steady state amounts of the $CF_1$-ATPase complex during development and ripening of tomato fruit as visualized by Western blot analysis. Lanes a to h are the developmental stages as described in "Materials and Methods," a to d are stages in fruit expansion, and d through h are stages in ripening; 1, 2, and 3 are the $\alpha$, $\beta$, and $\gamma$ subunits, respectively. Samples equivalent to 10 mg of fresh weight were subjected to SDS-PAGE and blot transferred to nitrocellulose as described under "Material and Methods."

The steady state levels of both the large and the small subunits of RuBPcase increased from stage a to c (Fig. 4). However, the small subunit increased at a much faster rate, by up to threefold, whereas the large subunit increased by only 150% over the same period (Table I). There was a gradual loss in the amounts of both subunits starting in stage c, but the large subunit still detectable in the final stages of senescence whereas the small subunit had completely disappeared (Fig. 4).

Immunoblotting with antibodies raised against chloroplast SOD revealed about a threefold increase in the enzyme during development and maturation (from stage a–c) and about a fourfold increase during ripening and senescence, after the transition from chloroplasts to chromoplast (Figs. 3 and 4; Table I). Surprisingly, high levels of SOD remained in the tissue during the late stages of senescence. The amount of PEPcase in the pericarp, as determined by Western blotting, was stable during the 'green' stages of fruit development, but the level increased during the later stages and reached its maximum concentrations late in the ripening process (Fig. 4).

## DISCUSSION

Immunological studies offer an effective approach for the study of the structure and organization of the photosynthetic apparatus in fruits. The use of quantitative immunolabeling enabled us to follow the sequential changes of the main chloroplastic polypeptides during the development of tomato fruits, in particular during the transition from chloroplast to chromoplast.

[1] Abbreviations: RuBPcase, ribulose-1,5 bisphosphate carboxylase; LSU, large subunit; SSU, small subunit; $CF_1$, chloroplast coupling factor; SOD, superoxide dismutase; PEPcase, phosphoenolpyruvate carboxylase; LHCP, light harvesting Chl protein.

Table I. *Changes in the Amounts of Various Polypeptides during Development and Ripening of Tomato Fruit*

The amounts are expressed as a percentage of the amount of the polypeptide at stage a. Quantitation was carried out as outlined in "Materials and Methods."

| Polypeptide | Stage | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | e | f | g | h |
| $CF_1$ | | | | | | | | |
| $\alpha$ | 100 | 110 | 132 | 117 | 113 | 119 | 10 | 9 |
| $\beta$ | 100 | 115 | 118 | 94 | 69 | 72 | 23 | 3 |
| $\gamma$ | 100 | 96 | 73 | 52 | 61 | 42 | 16 | 8 |
| $b_6/f$ | | | | | | | | |
| I | 100 | 85 | 96 | 80 | 53 | 44 | 26 | 26 |
| III | 100 | 115 | 117 | 24 | 23 | | | |
| PSII | | | | | | | | |
| 33 kD | 100 | 71 | 103 | 75 | 56 | 8 | | |
| 43 kD | 100 | 132 | 157 | 95 | 26 | | | |
| LHCP | 100 | 205 | 263 | 49 | 10 | | | |
| PSI | | | | | | | | |
| I | 100 | 75 | 78 | 121 | 128 | 106 | 109 | 58 |
| II | 100 | 140 | 143 | 60 | 12 | | | |
| III | 100 | 125 | 148 | 14 | | | | |
| VI | 100 | 121 | 191 | | | | | |
| VII | 100 | 98 | 151 | 45 | 22 | | | |
| RuBPcase | | | | | | | | |
| I | 100 | 114 | 158 | 85 | 123 | 90 | 51 | 9 |
| II | 100 | 252 | 305 | 207 | 194 | 57 | 10 | |
| PEPcase | 100 | 96 | 91 | 96 | 113 | 139 | 53 | 35 |
| SOD | 100 | 109 | 284 | 228 | 164 | 432 | 247 | 107 |

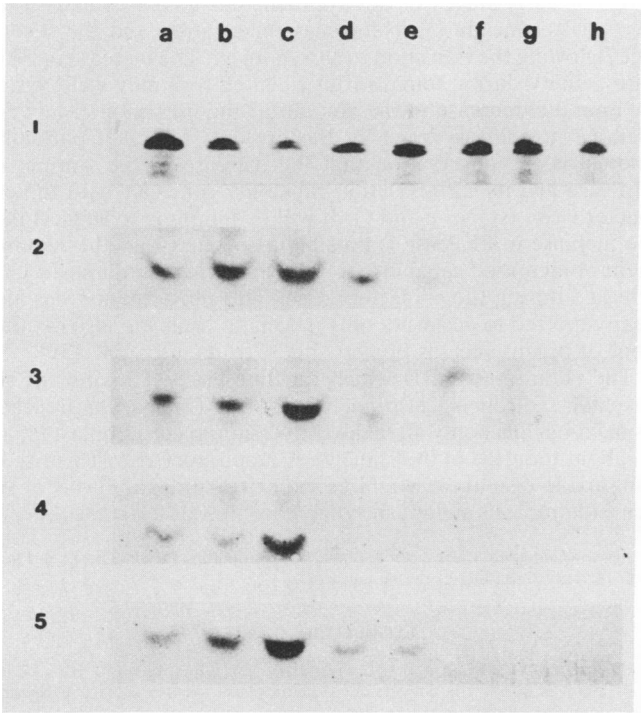


FIG. 2. Changes in the amounts of five subunits of the PSI complex during developmental and ripening stages of tomato fruits as visualized by Western blotting. Lanes a through h are as indicated in Figure 1. Panels 1 to 5 indicate blots reacted with different antibodies as follows: 1, 68 kD polypeptide of PSI complex (10 mg fresh weight) 2, subunit II (15 mg fresh weight); 3, subunit III (15 mg fresh weight); 4, subunit VI (15 mg fresh weight); 5, subunit VII (15 mg fresh weight).

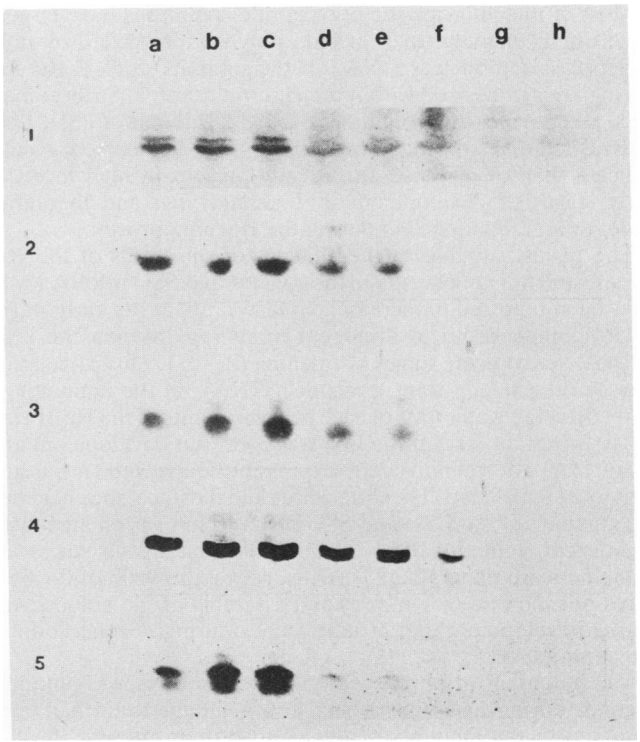


FIG. 3. Steady state levels of thylakoid proteins in tomato fruits at various stages of development and ripening (lanes a–h). 1, Cyt *f*; 2, Rieske protein; 3, 43 kD subunit of PSII; 4, 33 kD subunit of PSII; 5, LHCP of PSII.

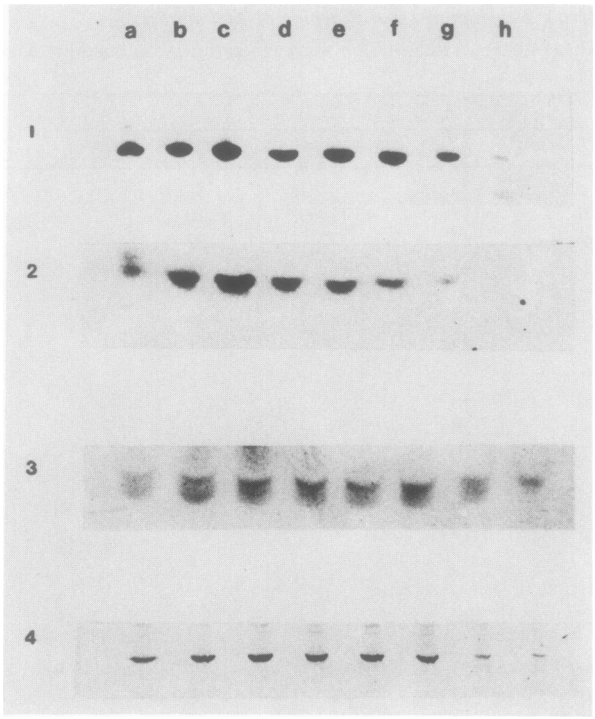


FIG. 4. Changes in the immunologically detectable amounts of RuBPcase, SOD, and PEPcase during development and ripening of tomato fruits. The stages (lanes a–h) are indicated in Figure 1. 1, LSU of RuBPcase; 2, SSU of RuBPcase; 3, SOD; 4, PEPcase.

Most of the chloroplastic polypeptide complexes have bi-genomic origin; namely, they include polypeptides coded by both chloroplast and nuclear DNA. Of the subunits studied, the following are synthesized within the chloroplast of 70 S ribosomes: P700 reaction center protein of PSI, 43 kD subunit of PSII, LSU of RuBPcase, $\alpha$ and the $\beta$ subunits of $CF_1$. The present results indicate that all of these are already present in high levels at early stages of development in *L. esculentum* and thereafter undergo a gradual decline during the ripening process.

The results summarize the changes of the levels of the RuBPcase and are noteworthy in the context that SSU mRNA levels have been reported to increase at a faster rate at the early stages of development and to disappear more rapidly than the LSU mRNA at advancing stages of ripening (22, 23). This correlation between the steady state levels of mRNA and the immunologically detectable amounts of each of the subunits of the RuBPcase suggests that, at least at the first stages of fruit development and at late stages of ripening, gene expression controls the levels and activity of RuBPcase. The differences and the imbalance between the amounts of the LSU and SSU mRNAs have been attributed to different regulation of nuclear and chloroplast gene expression in the pericarp tissue (22, 23). Thus, gene expression of the SSU may limit the rate of synthesis and assembly of the holoenzyme during development and at later stages during degeneration of the chloroplast.

The potential for photosynthetic electron transport generally declines during leaf senescence, and a similar trend has been observed in the amounts of thylakoid protein present (3, 25). The observation that mature tomato fruit pericarp exhibits maximal photosynthetic activity (27) is consistent with our observation that at this stage the immunologically detectable thylakoid polypeptides reach the highest level and decline thereafter.

The 68 kD polypeptide of PSI (SUI) was found to be at the highest level already at early stages of development and stayed constant throughout the developmental and ripening processes. Similarly, in etiolated leaves this P700 polypeptide is already present in proplastids, whereas all the other subunits of the PSI complex appear at later stages (19, 20).

The changes of LHCP correlate very well with the temporal pattern of the decline of LHCP mRNA during tomato fruit ripening (23). Thus, the fast disappearance of the LHCP at this stage can be attributed to an inactivation of the gene encoding for this protein.

This data reveal differences in the stability of the various subunits of the Cyt $b_6/f$ complex. Cyt $f$ was relatively stable, whereas the Rieske protein disappeared during the transition from chloroplast to chromoplast. In contrast to this process, during leaf senescence, Cyt $f$ was found to decrease at early stages and was suggested to constitute the rate-limiting factor of the photosynthesis (3, 25).

The parallel disappearance of Chl, PSI, PSII, and $b_6/f$ complex indicates that during the conversion of chloroplast to chromoplast, the capability for light harvesting, photochemical reactions, and electron transport is lost, and normal photosynthesis does not take place.

Doubts have been raised regarding the existence of net photosynthetic $CO_2$ fixation in tomato fruits (34). Our results indicate the presence of RuBPcase even at very late stages of ripening. However, it seems unlikely that the Calvin cycle is operable at this stage, since the main components of the light reactions are destroyed at earlier stages, and there is no supply of the light reaction products (ATP, NADPH) which are required for the dark reactions of photosynthesis.

It has been proposed that during the ripening process, the high activity of PEPcase enables the carboxylation and recycling of the respiratory $CO_2$ (2, 33, 34). The present study has provided evidence that the amount of PEPcase is already present at high levels at early stages of development but increases and shows two peaks, one during chloroplast maturation and the second one following the transition to chromoplast. The increase in PEPcase activity during tomato fruit ripening (34) may well be due to a parallel increase in the amount of the immunologically detectable protein as shown in the present study. Of particular interest is the observation that the increase in the amount of PEPcase and its carboxylation capability coincides with the climacteric rise (stages e and f). It will be tempting to suggest that the increase in PEPcase is possibly associated with the requirement of increased capability to recycle the high amounts of $CO_2$ released during the climacteric rise. This phenomenon has also been reported to occur not only in tomato fruits but also in other nonleaf organs such as flowers, buds, fruits, etc. (24, 33)

The chloroplast SOD which functions as a detoxifier of superoxide radicals has also been followed. This enzyme reached its maximal level only after the conversion to chromoplast (stage f), about the time of the climacteric respiratory rise. This rise of superoxide dismutase would be expected since the levels of superoxide radicals significantly increase in senescing tissues.

*Acknowledgments*—The authors thank Drs. Benjamin Horwitz and E. B. Dumbroff for critical reading of the manuscript.

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