Fibril Assembly and Carotenoid Overaccumulation in Chromoplasts: A Model for Supramolecular Lipoprotein Structures

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Chromoplast development in ripening bell pepper fruits is characterized by a massive synthesis of carotenoid pigments, resulting in their distinctive red color. We have shown that 95% of these pigments accumulate in chromoplasts in specific lipoprotein fibrils. In addition to carotenoids, purified fibrils contain galactolipids, phospholipids, and a single, 32-kD protein, designated fibrillin, which has antigenically related counterparts in other species. Fibrils were reconstituted in vitro when purified fibrillin was combined with carotenoids and polar lipids in the same stoichiometric ratio found in fibrils in vivo. Antibodies directed against fibrillin were used to isolate a fibrillin cDNA clone and, in immunological studies, to follow its accumulation during the chloroplast-to-chromoplast transition under different conditions. A model for fibril architecture is proposed wherein carotenoids accumulate in the center of the fibrils and are surrounded by a layer of polar lipids, which in turn are surrounded by an outer layer of fibrillin. Topological analysis of purified fibrils verified this structure. Collectively, these results suggest that the process of fibril self-assembly in chromoplasts is an example of a general phenomenon shared among cells that target excess membrane lipids into deposit structures to avoid their destabilizing or toxic effects. In addition, we have shown that abscisic acid stimulates this phenomenon in chromoplasts, whereas gibberellic acid and auxin delay it.

INTRODUCTION

Considerable study has been devoted to the classification of plant chromoplast types as either fibrillar, globular, reticulotubulous, membranous, or crystalloidal types according to their carotenoid-containing substructures (Sitte et al., 1980; Thomson and Whatley, 1980). Little is known about the reason for these various morphological types. In chromoplasts, threadlike fibrils were first postulated to be accumulation sites for excess carotenoids (Steffen, 1955; Steffen and Walter, 1955; Frey-Wyssling and Kreutzer, 1958). Similar fibrillar elements were subsequently described in sepals, petals, fruits, arils, and roots of more than 30 genera. Despite their occurrence, it was not known how this abundant accumulation of carotenoids was linked to the existence of fibrils.

In cells, excess lipids usually induce the formation of specialized subcellular lipoprotein elements to sequester those lipids. This occurs in prokaryotes and eukaryotes whose lipid levels increase due to metabolic deregulation. In animals, excess cholesterol stimulates the induction of macrophages that accumulate lipid bodies that become enriched in esterified cholesterol (Fruchart, 1992). Similarly, yeast (Clausen et al., 1974; Taylor and Parks, 1976) and tobacco (Maillet-Vernier et al., 1991; Schaller et al., 1992; Gondet, 1993) mutants that overproduce sterols spontaneously produce an abundance of lipid globules, which sequester the excess sterols. In seeds, excess triacylglycerols accumulate within oleosomes (Appelqvist, 1975), which consist of a triacylglycerol core surrounded by a layer of polar lipids that contain an oleosome-specific protein termed oleosin (Vance and Huang, 1987; Li et al., 1992; Tzen and Huang, 1992). Similar mechanisms may occur in chromoplasts.

Under normal circumstances, carotenoids in green plastids associate with cellular membranes. However, when produced in excess, such as in differentiating chromoplasts, carotenoids accumulate inside discrete plastid substructures. This is evident in tissues undergoing color changes associated with flower or fruit development; however, it also occurs in non-plant tissues. For example, carotene bodies have been described for fungi (Heim, 1946; Schranz, 1965; Eymé and Parriaud, 1970) and algae (Sprey, 1970; Ben-Amotz et al., 1982). In higher
plants, carotene bodies have been reported for plastids of carrot roots (Ben-Shaul and Klein, 1965) and pheasant's-eye flower petals (Kuhn, 1970). Likewise, lycopene bodies have been described for tomato chromoplasts (Ben-Shaul and Naftali, 1969; Harris and Spurr, 1969a, 1969b).

Evidence from comparative biochemical and structural studies indicates that the shape of these lipid-containing structures is governed by their lipid-to-protein ratio. A high proportion of lipid induces the formation of globular structures, whereas a high proportion of protein induces the formation of membranous or threadlike structures. Thus, fibril formation in chromoplasts may be governed by the proteins associated with carotenoid lipids. Support for this is provided by descriptions of chromoplast fibrils of Japanese rose (Wuttke, 1976), nasturtium (Winkenbach et al., 1976; Emter et al., 1990), and *Palisota barteri* (Knoth et al., 1986). A biochemical model for fibrillar structures was proposed for nasturtium (Winkenbach et al., 1976) and *P. barteri* (Knoth et al., 1986) in which the 30-kD protein is buried in the polar sheath and interacts with the core composed mainly of carotenoids. It was never determined whether this or antigenically related proteins were present in chromoplast fibrils of other species. Ultimately, a thorough understanding of the organization and the formation of chromoplast fibril requires topological studies, purification and characterization of fibril-associated proteins, and in vitro functional analysis.

Continuing our studies on chromoplast differentiation in bell pepper fruits (Camara et al., 1989; Kuntz et al., 1989), we have now coupled ultrastructural observations with biochemical and molecular findings to provide an explanation for fibril formation. In this study, we describe a procedure for fibril isolation and analysis and show that a specific protein, designated fibrillin, combines with polar lipids and esterified xanthophylls to form the fibrillar network observed in vivo and in identical structures reconstituted in vitro. In addition, we characterize a cDNA for the fibrillin gene and follow its expression in relation to other carotenoid biosynthetic genes during chromoplast differentiation and describe several features of its regulation in relation to hormone and chemical treatments. Our studies revealed that fibrillin is immunologically related to proteins described for fibrillar chromoplast fibrils from other species, which suggests a widespread role for fibrillin in plants.

**RESULTS**

In Situ Organization of Chromoplast Substructures

During ripening in bell pepper fruits, specific membrane vesicles and fibrils arise inside developing chromoplasts (Spurr and Harris, 1968; Suzuki, 1974; Simpson and Lee, 1976; Camara and Brangeon, 1981). This process begins when fruits reach their final stage of growth. In mature green fruits, only normal thylakoid membranes, typical of chloroplasts, were observed (Figure 1A). Upon ripening, however, the chlorophyllous thylakoid membranes became progressively disorganized (Figure 1B) and were replaced by a new membrane network. This was accompanied in orange or red fruits by a massive accumulation of plastoglobules that eventually elongated into fibrillar structures (Figure 1C). Fibrils and plastoglobules (Figure 1C) were easily distinguished from their surroundings by poststaining with silver proteinate (Thiéry, 1967). As in plastid envelope membranes, this positive staining reaction was caused by the presence of galactolipids in chromoplasts (Table 1).

In normal bell pepper varieties, the appearance of red carotenoid pigments is accompanied by the simultaneous loss of chlorophyll. One theory holds that during the chloroplast-to-chromoplast transition, chromoplasts obtain their new internal membrane components from degraded thylakoid membranes. This is inconsistent with observations in the bell pepper color mutant cultivar Sweet Chocolate, which derives its unusual pigmentation from carotenoids that develop in the presence of chlorophyll. Electron microscopy revealed that carotenoid-containing fibrils and internal membranes arise in the presence of chlorophyll-containing thylakoids within the same plastid (Figure 1D). Thus, thylakoids do not degrade but remain well preserved despite carotenoid accumulation and fibril formation. This suggests that fibril formation does not rely on materials released from degraded thylakoid membranes. Fibrils are likely synthesized de novo, in agreement with previous data (Laborde and Spurr, 1973).

**Isolation and Characterization of Chromoplast Fibrils**

In light of our observations, we developed a procedure for purifying chromoplast fibrils, as described in Methods. Electron microscopy of purified fractions showed isolated chromoplast fibrils (Figure 2A) estimated to be 95% pure. The characteristic shape of purified fibrils was identical to that observed for fibrils in vivo, and analyses of cross-sections of purified fibrillar fractions revealed the presence of cross-bridges (Figure 2B). Fibril cores appeared as empty zones surrounded by a dense outer layer. This was likely caused by osmium tetroxide staining, which reacts primarily with polar lipids (Bahr, 1954; Sitte, 1976; Winkenbach et al., 1976) of the outer layer, whereas the more apolar carotenoids in the center core are solubilized and removed during sample processing.

**Lipid and Protein Components of Chromoplast Fibrils**

To study the spatial organization of chromoplast fibrils, we analyzed the distribution of their protein, polar (galactolipids and phospholipids), and apolar (carotenoids and tocopherols) lipid components (Tables 1 and 2). Qualitatively, all categories of polar lipids and carotenoids described previously for isolated bell pepper chromoplasts (Camara and Mönéger, 1978; Camara et al., 1983) appear in purified fibrils. The high level of galactolipids (monogalactosyldiglyceride and digalactosyldiglyceride) also agrees with the observed reactivity of fibrils and their
Figure 1. Electron Micrographs Showing Ultrastructural Changes during Chloroplast-to-Chromoplast Differentiation in Bell Pepper Fruits.

(A) Chloroplast in mature green fruit. Bar = 1 μm.
(B) Chloroplast during early steps of thylakoid disintegration showing the formation of plastoglobules. Bar = 1 μm.
(C) Fully differentiated chromoplast showing intense accumulation of fibrils (arrow) and plastoglobule-associated fibrils. These latter structures were poststained with silver proteinate. Bar = 1 μm.
(D) Fully differentiated chromoplast from the bell pepper cultivar Sweet Chocolate color mutant showing a persistent thylakoid system (asterisks) and a network of fibrils and plastoglobule-associated fibrils in the same plastid (arrow). Bar = 1 μm.
Table 1. Lipid Content of Isolated Chloroplast Thylakoids and Chromoplast Fibrils

<table>
<thead>
<tr>
<th>Lipids</th>
<th>Chloroplast Thylakoids (nmol/mg Protein)</th>
<th>Chromoplast Fibrils (nmol/mg Protein)</th>
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</thead>
<tbody>
<tr>
<td>PL^a</td>
<td>150</td>
<td>70</td>
</tr>
<tr>
<td>MG^b</td>
<td>350</td>
<td>900</td>
</tr>
<tr>
<td>DG^c</td>
<td>250</td>
<td>460</td>
</tr>
<tr>
<td>CAR^d</td>
<td>50</td>
<td>1000</td>
</tr>
<tr>
<td>TOC^e</td>
<td>6</td>
<td>70</td>
</tr>
<tr>
<td>CAR/PL + MG + DG</td>
<td>0.06</td>
<td>0.70</td>
</tr>
</tbody>
</table>

^a Total phospholipids.
^b Monogalactosyldiacylglycerol.
^c Digalactosyldiacylglycerol.
^d Total carotenoids.
^e Total tocopherols.

associated plastoglobules with silver proteinate staining, as mentioned previously (Figure 1C). Quantitatively, fibrils contain 95% of the total carotenoids present in chromoplasts and most of these are esterified (Table 2). The lipid-to-protein ratio (Table 1) of chromoplast fibrils was significantly different from the lipid-to-protein ratio of chloroplast thylakoids. These results permit chromoplast fibrils to be classified as lipoprotein elements according to Scanu (1972).

SDS-PAGE of chromoplast membrane fractions revealed the presence of an abundant, ~32-kD polypeptide in fibrils. This polypeptide, termed fibrillin, was similar in size to proteins from fibrils of Japanese rose (Wuttke, 1976), nasturtium (Winkenbach et al., 1976; Emter et al., 1990), and P. barteri (Knoth et al., 1986). Fibrillin could not be labeled with N-(ethyl-1,2-^14C) ethylmaleimide. This suggests the absence of free thiol or cysteine groups in its primary protein structure.

Purification and Characterization of Fibrillin, the Major Protein Associated with Chromoplast Fibrils in Vivo

Based on the above data, we devised a procedure for the purification of fibrillin, as described in Methods. Affigel-501 proved to be convenient for purification because the absence of free-thiol groups in fibrillin allowed the flow-through of fractions highly enriched in fibrillin and retained other thiol-containing proteins. The final purification yielded a single band of ~32 kD after SDS-PAGE (Figure 2C).

Figure 2. Electron Micrographs of Isolated Fibrils and Electrophoretic and Immunological Analysis of Fibril Proteins.

(A) Longitudinal section of the fraction containing purified fibrils. Bar = 0.5 μm.
(B) Transverse section through the fraction containing the purified fibrils showing the presence of cross-bridge structures (arrow). Bar = 0.1 μm.
(C) SDS-PAGE analysis of fibrillin during different purification steps. Lanes shown from left to right represent crude chromoplast membranes (lane 1), isolated fibrils (lane 2), unpooled (lane 3) and pooled (lane 4) fractions after chromatofocusing on PBE 94 (Pharmacia). The position of fibrillin, the major fibril protein, is indicated (arrow). Molecular mass is indicated in kilodaltons at left.
(D) Identification of fibrillin by immunoblot analysis. Purified fibrillin (lane 1), total chloroplast proteins (lane 2), chromoplast membrane proteins (lane 3), and chromoplast stromal proteins (lane 4) were probed with anti-fibrillin antibody and a phosphatase-conjugated second antibody to reveal the immunocomplex band, which is indicated by the arrow.
Table 2. Carotenoid Composition of Isolated Chromoplast Fibrils

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Composition (%)</th>
<th>Esterification (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene</td>
<td>4.0</td>
<td>—</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>1.0</td>
<td>—</td>
</tr>
<tr>
<td>ζ-Carotene</td>
<td>3.2</td>
<td>—</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>6.4</td>
<td>—</td>
</tr>
<tr>
<td>β-Cryptoxanthin</td>
<td>5.1</td>
<td>75</td>
</tr>
<tr>
<td>Cryptocapsin</td>
<td>2.5</td>
<td>90</td>
</tr>
<tr>
<td>Lutein</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Zeaxanthin</td>
<td>4.5</td>
<td>95</td>
</tr>
<tr>
<td>Antheraxanthin</td>
<td>2.6</td>
<td>90</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>6.5</td>
<td>88</td>
</tr>
<tr>
<td>Capsanthin</td>
<td>54.7</td>
<td>95</td>
</tr>
<tr>
<td>Capsanthin epoxide</td>
<td>0.5</td>
<td>90</td>
</tr>
<tr>
<td>Capsorubin</td>
<td>6.0</td>
<td>90</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>2.0</td>
<td>87</td>
</tr>
</tbody>
</table>

* The proportion of esterified xanthophylls was determined as described in Methods.

Chromoplast fibril assembly was further characterized by immunological methods. It was not found in chloroplasts isolated from leaves or green fruits and could only be detected in chromoplasts. Protein gel blotting of purified, total chromoplast membrane fractions yielded a single immunocomplex band (Figure 2D). Small amounts of fibrillin were also detected in chromoplast stroma (Figure 2D). This altered form of fibrillin was either a non-membrane-associated form that did not sediment after 1 hr at 100,000g or a soluble precursor of fibrillin that appeared prior to its integration into fibrils.

Indirect immunofluorescence staining of pericarp tissue from orange fruit using fibrillin-specific antibodies indicated a strong positive reaction that was confined to the plastid compartment (Figure 3A). Immunogold labeling confirmed that this protein was localized in isolated fibrils (Figures 3B and 3C).

Carotenogenic organs of nasturtium and Cucurbita maxima containing fibrillar chromoplasts were also examined. Proteins probed with fibrillin-specific antibodies showed an immunocomplex band of 32 kD in nasturtium flower petals. In C. maxima flower and fruit, one major band at 67 kD and one minor band at 43 kD were also observed. These data indicated that bell pepper fibrillin has antigenic determinants identical to those of nasturtium and C. maxima. The difference in molecular mass between bell pepper fibrillin and the C. maxima proteins probably does not affect their structural roles. Similar observations have been made (Tzen et al., 1990) for oleosins, which also show large variations in molecular mass.

Topological Analysis of Lipid Components and Fibrillin in Isolated Chromoplast Fibrils

To analyze the topological distribution of lipids and proteins within fibrils, an assay was developed based on the accessibility of carotenoids to solvents of differing polarities following phospholipase or trypsin treatments. A series of extractions using solvents with increasing water solubilities was used to extract fibril preparations with hexane (which can absorb 0.02% water [v/v]), chloroform (0.5% water), diethyl ether (7.5% water), or acetone (which is totally water soluble).

Lipid accessibility was monitored by absorbance at 480 nm due to the release of carotenoids into the solvent. Under these conditions, hexane, chloroform, and ether failed to extract the bulk of the carotenoids, as shown by the low absorbance at 480 nm, in contrast to acetone (Figure 4A). Lipid accessibility to solvents was then examined following phospholipase treatment to test the possible role of polar phospholipids in

![Figure 3. Immunocytochemical Analysis of Fibrillin Compartmentation in Pericarp Tissue of Red Bell Pepper.](image)

(A) Immunofluorescent detection of fibrillin. Fixed sections were incubated with anti-fibrillin antibodies followed by a fluorescein-conjugated secondary antibody to identify specific and intense labeling in the chromoplast compartments.

(B) Electron micrograph analysis of fibrillin in isolated fibrils after immunogold labeling. A suspension of isolated fibrils was treated with preimmune serum as described in Methods.

(C) Electron micrograph analysis of fibrillin in isolated fibrils after immunogold labeling. A suspension of isolated fibrils was treated with anti-fibrillin antibodies as described in Methods.
maintaining fibril architecture. When isolated fibrils were incubated with phospholipase A$_2$ or C, no additional release of carotenoid into the organic solvent was observed, compared to control preparations devoid of phospholipase. Additional analysis also revealed that no significant hydrolysis of phospholipids occurred under the incubation conditions used (data not shown). Lipid accessibility was then examined following protease treatments to test the involvement of proteins in fibril structure. When fibrils were incubated with trypsin, a time-dependent release of carotenoids could be observed into all solvents including diethyl ether (Figures 4A and 4B). Immunological analysis of the proteolytic fragments using fibrillin-specific antibodies revealed that the release of carotenoids coincided with the degradation of fibrillin (Figures 4B and 4C). Moreover, when isolated fibrils were incubated together with trypsin and trypsin inhibitor, the release of carotenoids was inhibited (Figures 4B and 4C). Finally, when trypsin treatments preceded phospholipase treatments, phospholipids were hydrolyzed (data not shown) compared to phospholipase treatments alone (see above).

These data suggested that carotenoids and polar lipids of chromoplast fibrils are protected by an outer layer of protein composed almost exclusively of fibrillin. This is analogous to the structure postulated for oil bodies by Tzen and Huang (1992), who used similar approaches to show that a phospholipid-protein complex surrounds a triacylglycerol core. Unlike oil bodies, fibrils possess carotenoids and galactolipids and have higher protein and lower phospholipid concentrations.

**Role of Fibrillin and Cyclic Carotenoids in Reconstituting Chromoplast Fibril Morphotypes in Vitro**

To delineate a path for the molecular assembly of fibrils into linear structures, the contribution of different fibril components was analyzed in reconstitution assays. Fibrils were reconstituted in vitro by slowly adding fibrillin into a mixture of polar and isoprenoid lipids that occur in authentic fibrils. Under these conditions, fibril formation was strictly dependent upon fibrillin concentration (Figure 5A). Fibrils did not form when other components were substituted. For example, when triacylglycerol replaced the polar lipids, oil droplets formed instead of fibrils. Likewise, if bovine serum albumin, a protein known to bind carotenoids, replaced fibrillin then fibril assembly did not occur. Fibrils only occurred when the lipid-to-protein ratio approached that of in vivo fibrils (Figure 5A).

The involvement of cyclic carotenoids in fibril assembly was also examined in reconstitution assays. We compared the effect of lycopene (acyclic) and cyclic carotenoids including β-carotene and several different xanthophylls (zeaxanthin, zeaxanthin diester, capsanthin, and capsanthin diester) in reconstituting fibrils. When lycopene was used, fibril assembly was blocked, in contrast to cyclic carotenoids, which all allowed fibril assembly (Figure 5B). We observed that β-carotene
Figure 5. In Vitro Reconstitution of Fibrils with Purified Fibrillin and Different Lipid Components.

(A) Isolated fibril carotenoids (Car) and polar lipids (monogalactosyl-diacylglyceride, digalactosyldiacylglyceride, and phospholipids) were qualitatively and quantitatively used in a molar ratio identical to those described in Table 1. In the case of triacylglycerol, trilein was added in place of and in the same molar ratio as polar lipids. Purified fibrillin was used as indicated, prior to determining the extent of fibril reconstitution as indicated in Methods.

(B) Effect of structurally different carotenoids on the reconstitution of fibrils in vitro. The reconstitution procedure was as described in Figure 5A, except that purified carotenoids were used as indicated.

(C) Electron micrograph of fibrils reconstituted in vitro. Isolated fibril carotenoids and polar lipids, qualitatively and quantitatively identical to those described in Table 1, were used to reconstitute fibrils with purified fibrillin, using 1.43 nmol of total lipids (galactolipids, phospholipids, and carotenoids) per mg of pure fibrillin as described in Methods. Bar = 0.7 μm.

Effect of Carotenoid Bioregulator on Fibril Assembly in Vivo

Chlorophenylthiotriethylamine (CPTA) is known to disrupt normal chloroplast development (Coggins et al., 1970), to induce the formation of lycopene, and to suppress the synthesis of β-carotene and its oxidized derivatives. When intact, mature, green fruits were treated with 0.1 M CPTA, noticeable structural changes occurred in plastids, resulting in the formation of lycopene crystals with a concomitant absence of fibrils (data not shown). This is due either to the destabilization of membranes by excess lycopene (Walles, 1971; Simpson et al., 1974) or to the lack of cyclic carotenoids caused by CPTA treatment. Similar observations occurred in flower petal chromoplasts when carotenoids were blocked at the level of phytoene (Emter et al., 1990).

To examine whether CPTA affects fibrillin accumulation, we made use of the in vitro-cultured pericarp tissues from bell pepper. Under these conditions, the time-dependent accumulation of lycopene was monitored in relation to appearance of fibrillin using fibrillin-specific antibodies. Results indicated that fibrillin as well as a high molecular mass form of fibrillin accumulated in the presence of CPTA. Analysis by in vitro transcription/translation of full-length fibrillin cDNA indicated that this larger form corresponded to the predicted molecular weight of a fibrillin precursor (data not shown). This suggested that CPTA blocked the translocation or maturation of this plastid-directed protein in addition to its other known roles in carotenogenesis, but it did not block the synthesis of fibrillin.

cDNA Cloning and Developmental Expression of Chromoplast Fibrillin

Screening of a bell pepper library (Kuntz et al., 1992) was performed initially using fibrillin-specific antibodies and then using...
The cloned fibrillin cDNA encodes a protein with a predicted molecular mass of 35.2 kD (Figure 6A) whose N-terminal end possesses features similar to the transit sequence from another bell pepper chromoplast protein, cysteine synthase (Römer et al., 1992). It seems likely that the acidic motif DKEDE at position 64 is not part of the transit peptide and that the cleavage site is located before this sequence. Thus, the putative transit peptide is comprised of ~60 amino acids, and the mature protein has a predicted molecular mass of ~29 kD and a predicted pI of 4.5. The fact that the mature protein does not contain any cysteine residues agrees with the observed lack of thiol groups in purified fibrillin. Also, the deduced fibrillin sequence displays several tandem aspartic and/or glutamic acid residues at positions 66 to 68, 85 to 87, 175 to 176, and 222 to 223, which are likely to be surface exposed and may explain why both isolated chromoplast fibrils and purified fibrillin precipitate in the presence of calcium (B. Camara, unpublished results). A search through the protein data bank revealed no significant homology to previously published amino acid sequences. However, the sequence RGD, which is a typical cell adhesion motif (D’Souza et al., 1991), was found at position 303. The hydropathy profile of fibrillin (Figure 6B) indicates a region of high hydrophilicity in the N terminus, while most of the hydrophobic regions are located in the middle and the C-terminal portions of the protein.

The fibrillin cDNA clone was used as a hybridization probe in RNA blot experiments (Figure 7). No hybridization signal was detected (even after overexposure of the autoradiograph) in RNA extracted from developing leaves or senescing leaves. In RNA from fruit at various developmental stages, a faint signal was detected in mature green fruit immediately before the onset of ripening but not in earlier green stages. The hybridization signal increased incrementally as fruit ripened and reached its greatest level at the fully mature stage at the height of color development. A different pattern of induction was observed for geranylgeranyl pyrophosphate synthase (Kuntz et al., 1992) and phytoene synthase (Römer et al., 1993). In addition, unlike fibrillin, phytoene–phytofluene desaturase (Hugueney et al., 1992) gene expression was practically constitutive. Based on these observations, we concluded that the fibrillin gene and these carotenogenic genes are most likely not under the control of the same regulatory mechanisms, despite the accumulation of each of these proteins during the period of chromoplast differentiation.

When RNAs were examined from tomato fruits at various green or ripening stages, no hybridization signal to the fibrillin gene could be detected in any of the RNA samples (data not shown). This confirmed our observation that fibrillin has no counterpart in tomato chromoplasts (data not shown) that possess crystalline-type chromoplasts and accumulate lycopene rather than cyclic carotenoids.

Immunological experiments revealed that fibrillin was not detected at any stage of early fruit growth in bell pepper (data not shown). Fibrillin accumulation was noticeable only during the period of chromoplast differentiation. Therefore, fibrillin is a typical chromoplast protein as has been shown for a 58- and a 35-kD protein designated ChrA and ChrB (Hadjeb et al., 1988; Newman et al., 1989). In the fruit color mutants Alma, Sweet Orange, Jaune de Pignerolle, and Sweet Chocolate (see Methods), ripening is characterized by a massive deposition of epoxy-xanthophylls or keto-xanthophylls. Also in these mutants, fibrillin was not detected during fruit growth but only during chromoplast differentiation (data not shown). Because thylakoid structure was preserved in the cultivar Sweet Chocolate (Figure 1D), the presence of fibrillin in this mutant indicated...
Figure 7. RNA Gel Blot Analysis Showing Expression of Fibrillin Gene in Leaves and Fruits at Different Stages.

(A) Total RNA was isolated from young (lane 1) and senescing (lane 2) leaves, and 7 μg of RNA was loaded in each lane. The RNA gel blots were hybridized with a cDNA encoding a 25S ribosomal RNA to ensure that equivalent amounts of RNA were loaded onto the gel.

(B) Total RNA was isolated from fruits at immature stages corresponding to a pericarp diameter of less than 1 cm (lane 1), 3.5 cm (lane 2), at the mature green stage (lane 3), at the early ripening stage (lane 4), and at the red stage (lane 5). The experimental conditions are as given in (A).

that fibrillin synthesis is independent of thylakoid degradation. On the other hand, the reduction of bicyclic carotenoid accumulation induced by CPTA was paralleled by a drop in the content of plastid fibrillin (Figure 8).

Hormonal effects on fibrillin accumulation were monitored immunologically during in vitro differentiation of pericarp tissues. Pericarp of mature green fruits was allowed to ripen, as described in Methods, in the presence of several hormones at different concentrations as indicated in Figure 9C. In untreated pericarp, fibrillin synthesis was apparent after 3 to 5 days and increased until 15 days, at which time chromoplasts were fully differentiated, as indicated by the complete disappearance of chlorophylls (results not shown) and the synthesis of keto-carotenoids (Figures 9A and 9B). Abscisic acid slightly accelerated fibrillin synthesis, while ethephon, a progenitor of ethylene, had little effect (Figure 9C). The same trend was also observed for carotenoid accumulation during the early ripening period (data not shown). In contrast, gibberellic acid and indoleacetic acid delayed synthesis of fibrillin and its associated chromoplast carotenoids for 2 weeks (Figure 9C). Kinetin had no apparent effect on fibrillin accumulation as compared to untreated pericarp tissue. The responses to hormone treatments were similar using concentrations of 10 μM to 1 mM.

DISCUSSION

It has been known for some time that chromoplasts of pepper display a strong birefringence and dichroism (Frey-Wyssling and Kreutzer, 1958) due to the internal carotenoids that are maintained in a parallel orientation within the chromoplasts. Our structural and biochemical data indicated that these carotenoids are not randomly dispersed or freely accessible within chromoplasts but are sequestered within specific substructures, the fibrils. These are aligned along the longitudinal axis of chromoplasts, which explains their birefringence. From our studies, a model for fibril architecture can be proposed.

The major components of fibrils are their bicyclic carotenoids, which occupy the fibril core. The core is surrounded by polar galactolipids and phospholipids, which orient their hydrophobic tails toward the carotenoids and their polar head groups outward toward the surface, which is composed of fibrillin (Figure 10). This model implies that the precursors of carotenones, such as phytoene, phytofluene, or ζ-carotene (Camara and Monte, 1982), once incorporated into fibrils, probably no longer act as intermediates in the biosynthetic pathway and are probably "dead-end" products. Unlike previously proposed models (Winkenbach et al., 1976; Knoth et al., 1986; Emter et al., 1990), our data suggest that fibrillin does not interact directly with the carotenoid core but only interacts with the polar lipids surrounding the core and, thus, acts as a shield in addition to its role in governing the shape of fibrils.

We have isolated fibrillin and have shown that it is involved in the packaging and organization of excess carotenoids in both in vivo and in vitro systems. One apparent question is whether fibrillin has only a structural role or also possesses enzymatic activity. We have been unsuccessful in attempts to

Figure 8. Protein Gel Blot Analysis of Fibrillin in Fruits at Different Ripening Stages.

Mature fruits were selected at different stages during the ripening period according to their color, which varied from green (lane 1), green-brown (lane 2), brown (lane 3), dark brown (lane 4), orange (lane 5), deep orange (lane 6), and red (lane 7), except in (lane 8) where the intact fruit was treated with 0.1 M CPTA and instead developed a yellow color after complete ripening. Total plastid protein (50 μg) was loaded in each lane. Immunodetection was performed as indicated in Figure 2D.
Figure 9. Effect of Hormones on Fibrillin Formation during in Vitro Ripening of Excised Pericarp Discs.

(A) Time course synthesis of keto-carotenoids during in vitro ripening in the absence of external hormone supply. Discs were removed for analysis after 0, 3, 5, 10, and 15 days.

(B) Immunoblot analysis of fibrillin during in vitro ripening in the absence of external hormone supply. Discs were removed as given in (A).

(C) Immunoblot analysis of fibrillin following hormone treatments after 5 and 15 days. Discs were treated with the indicated hormones using 100 μM concentrations except ethephon, which was used at 1 mM. CT, control; GA, gibberellic acid; IAA, indoleacetic acid; K, kinetin; ABA, abscisic acid; ET, ethephon.

Figure 10. Model of Chromoplast Fibril Assembly (not to scale).

The core is occupied by carotenoids (CAR) that interact with the acyl residues of the more polar galactolipids and phospholipids (LIP), whose polar head groups (dark circles) interact with fibrillin molecules (FIB), which are directly in contact with the plastid stroma.

is noteworthy that during evolution, enzymes can be altered to become structural proteins, as shown for cholinesterase, which may be involved in cell interactions in addition to its well-known hydrolytic function (Krejci et al., 1991).

Our reconstitution system is novel because the phenomenon occurs according to the stoichiometric self-assembly of carotenoids, galactolipids, phospholipids, and fibrillin. Such a reconstituted membrane system is unique for plant cellular structures. The carotenoid-containing fibrils that result have unexpected physical properties. Carotenoids, being buoyant and hydrophobic, normally float in a two-phase system with water. However, when sequestered within reconstituted fibrils, they form a particulate suspension that sinks in aqueous media. Carotenoids mixed with polar lipids alone form spherical lipid vesicles that are also buoyant. Fibrillin-mediated assembly is specific, because chloroplast proteins (Huang et al., 1973) or BSA, which is known for its capacity to bind carotenoids (Takagi et al., 1981), cannot induce fibril formation (data not shown). Instead, BSA emulsifies carotenoids with its aqueous medium, and the resulting dispersion exhibits none of the ordered structural features of reconstituted fibrils.

Two underlying questions are: Why do carotenoid-sequestering substructures occur in chromoplasts and is their formation essential for plastid (or cell) survival? Although several carotenoid-associated proteins have been described (Ke, 1971; Bullerjahn and Sherman, 1986; Masamoto et al., 1987; Cervantes-Cervantes et al., 1990; Milicua et al., 1991; Bryant et al., 1992; Markwell et al., 1992), their function in plastids is unclear. It is known that low levels of carotenoids are essential for the light-harvesting mechanism of chloroplasts (Plumley and Schmidt, 1987; Humbeck et al., 1989; Herrin et al., 1992), demonstrate a direct involvement of fibrillin in carotenoid synthesis. This is supported by immunological analysis that shows its absence in green fruit and leaf tissues in which carotenoids are known to occur, which implies that fibrillin's role is largely structural and that it is responsible for the linear arrangement of lipochromes into fibrils. This structural role is supported by reconstitution assays that demonstrate in vitro fibril assembly by the simple addition of fibrillin to a mixture of carotenoids and polar lipids. Although fibrillin is believed to be a structural protein, an enzymatic function for it cannot be discounted. It
and it has also been noted that inhibition of carotenoid synthesis can inhibit transport of nuclear-encoded proteins destined for the plastid (Dahlin, 1993). Our data showed that disruption of carotenoid synthesis following CPTA treatment also influences membrane function and affects the uptake and maturation of at least one nuclear-encoded plastid protein.

Excess carotenoids in pigmented flowers and fruits are necessary to ensure successful reproductive development of the species. However, to create tissues having the brilliant colors found in red pepper fruits, it is necessary to accumulate extraordinarily high concentrations of these lipid pigments. Such high lipid levels could disturb normal cellular functions (Siegel et al., 1981) and could be detrimental. It is well known that carotenoid overaccumulation results in the loss of photosynthetic capacity in ripening fruit. Therefore, mechanisms that permit the nondetrimental overaccumulation of such lipidic components must exist. In carotenogenic tissue, specific structures that sequester excess carotenoids have been described (Frey-Wysling and Kreutzer, 1958). The simplest is the globule that has a high total lipid-to-protein ratio (Hansmann and Sitte, 1982). In crystalline-type chromoplasts, lycopene crystals are sequestered into membrane structures. These are observed in tomato chromoplasts (Harris and Spurr, 1969b) or lycogenic maize mutants (Walles, 1971). In fibrillar chromoplasts (Winkenbach et al., 1976; Knott et al., 1986; Emter et al., 1990; this study), the presence of high amounts of fibrillin causes the ratio to drop considerably below that observed in globular types.

The molecular mechanisms behind fibril self-assembly are not understood. Electron microscopy suggests that in the initial steps of fibril formation in vivo, plastoglobules appear to play a key role as initiation sites for incipient fibril formation. This has been observed in chromoplasts in fruits of bell pepper (Simpson et al., 1977a), Japanese rose (Steffen and Walter, 1955), Jerusalem cherry (Steffen and Walter, 1958), and R. barteri (Knott et al., 1986), and in sepal of yellow water lily (Gronegress, 1974), and craneflower (Simpson et al., 1975), in which globules elongate into fibrils during the initial period of chromoplast differentiation; in waxwork arils (Bornman, 1968), and in asparagus fruits (Simpson et al., 1977b), where this is delayed until late in chromoplast differentiation. Fibril initiation occurs only with chromoplast plastoglobules and not with chloroplast plastoglobules that appear during senescence, mineral deficiency, herbicide treatment, or other stress conditions.

Isolation of the cDNA for fibrillin revealed that fibrillin is a nuclear-encoded protein with a transit peptide for chloroplast targeting. Consequently, the fact that fibrillin exists as a minor soluble form in addition to its more abundant, membrane-associated form suggests that fibrillin may be imported into the plastid stroma as a soluble protein before it participates in fibrils. The equilibrium between these two forms of fibrillin may be controlled by the production of lipid components, which, upon reaching a critical concentration, spontaneously recruit the fibrillin from the soluble pool. This mechanism is supported by the lipid dependence for fibril formation observed in vitro (Figure 5) and by the fact that fewer fibrils accumulated in orange and yellow fruits, which have lower carotenoid content than red fruits (results not shown). Also, studies with transgenic tobacco that express fibrillin show normal chloroplasts because carotenoid content is not increased (S. Römer, unpublished results).

The appearance of two chromoplast-specific proteins, designated ChrA and ChrB, has been documented previously (Hadje et al., 1988; Newman et al., 1989). Similarity in size between fibrillin and the 35-kD membrane protein ChrB, as well as in their time course of appearance, raises the possibility that these two proteins are related or identical. Availability of a ChrB sequence will clarify this point.

Features of the fibrillin amino acid sequence are consistent with its potential to interact with lipid components. Several hydrophobic regions exist that could be involved with lipid binding and the RGD sequence motif may account for the observed ultrastructural cross-bridges (Figure 2B). The RGD sequence is a conserved bonding motif for several adhesion proteins in animals (D’Souza et al., 1991) and raises the possibility that the alignment of fibrils observed in vivo could be due to this motif.

Physiological evidence indicates that there is some coordination between fibrillin and carotenoid biosynthetic genes during chromoplast differentiation. The mechanism by which these events are regulated is unknown. Fibrillin gene expression apparently precedes lipochrome overaccumulation; this suggests that carotenoids probably do not induce fibrillin gene expression directly.

METHODS

Plant Material

Pepper plants (Capsicum annuum cv Lamuyo) were grown under standard greenhouse conditions until fruits ripened (characterized by a red color). Other fruit color mutants were grown under the same conditions and include the following: cultivar Sweet Chocolate, which has a chlorophyll retainer gene and remains chocolate colored at the final ripening stage; cultivar Alma, in which the pale yellow mature fruit develops a red color at the final stage of ripening; cultivar Jaune de Pignerolle, in which the mature green fruit develops an orange color at the final stage of ripening. Flower petals of nasturtium (Tropaeolum majus) and flower petals and fruit of Cucurbita maxima were obtained from the University of Strasbourg Botanical Gardens.

Chromoplast Differentiation In Vitro

Chromoplast differentiation was monitored in vitro using the procedure of Campbell et al. (1990). Pericarp discs (1 cm diameter) were excised from mature green fruits previously surface sterilized with sodium hypochlorite, 70% ethanol, and several washes of sterile water. Discs (eight per treatment) were placed epidermis-side-down into wells
of a 24-well tissue culture plate (Corning) containing water. The culture plate was sealed with Urgopore (Strasbourg, France) and placed under a 12-hr 24°C/12-hr, 20°C, light/dark regime. Under these conditions, green pericarp discs of pepper tissues turned red within 15 days. Test solutions containing 10 μL of 100 mM indoleacetic acid, abscisic acid, gibberellic acid, kinetin, and ethephon dissolved in ethanol/methylsulfoxide/water were applied to the pericarp disc before incubation to observe differences in color changes with time.

Plastid and Subplastid Fractionation and Purification

Capsicum chromoplasts and chloroplasts were isolated and purified as described previously (Camara, 1985a, 1985b). Chloroplast thylakoids were isolated from purified chloroplasts according to the method of Douce et al. (1973). Fibrils were isolated from the plastid pellet obtained from 1 kg of red fruit pericarp that was subjected to a gentle osmotic shock by homogenization in the presence of 100 mL of Tris buffer, pH 7.6, containing 1 mM DTT (buffer A). The resulting suspension was loaded (20 mL per tube) onto the top of a discontinuous gradient (5 mL for each layer) consisting of 0.5 and 0.9 M sucrose in buffer A supplemented with 1 mM EDTA and centrifuged at 70,000g for 60 min at 4°C, using a rotor (model SW27, Beckman, Strasbourg, France). Following centrifugation, the fraction above the 0.8 M sucrose layer was retained and centrifuged again for 30 min at 100,000g. The resulting pellet was resuspended in buffer A (15 mL final volume) and loaded (5 mL per tube) onto the top of a 0 to 1 M linear sucrose gradient (25 mL per tube). After centrifugation at 70,000g for 12 hr at 4°C using a SW27 rotor, the purified fibril fraction was recovered at a density corresponding to 1.07 g/mL.

Purification of Fibrillins

The fibril suspension isolated from 2 kg of fruit pericarp, as described above, was treated with an equal volume of 50 mM Tris buffer, pH 7.6, containing 2% Triton X-100 (buffer B). After stirring for 30 min at 4°C and centrifugation at 100,000g for 1 hr, the resulting supernatant was used directly or stored at −70°C. As a first step in the purification of fibril, the supernatant was dialyzed against buffer B to remove any trace of DTT before loading onto an Affigel-50 column (2.5 × 25 cm) equilibrated with buffer B. The flow-through fraction was saved and loaded onto a Q-Sepharose (Pharmacia) column (2.5 × 25 cm) equilibrated with buffer B. Fibrillins were eluted as the major protein peak, with a linear gradient of 0 to 0.3 M NaCl in buffer B. Finally, the fibrillin containing fractions were adsorbed onto a PBE 94 (Pharmacia) column (2 × 20 cm) equilibrated with 25 mM imidazole-HCl buffer, pH 7, before elution with a linear pH (7 to 3.8) gradient using Polybuffer 74. The protein peak eluting between pH 5 to 4 was retained.

cDNA Library Screening and DNA/RNA Techniques

Screening and DNA/RNA techniques were performed as described previously (Kuntz et al., 1992).

Topological Analysis of Fibril Lipid Components

To analyze the topological organization of the different fibril lipid components, their accessibility to different solvents was determined before and after treatment with phospholipase A2 and C or trypsin in the presence and absence of trypsin inhibitor. For our test, 500 μL of purified chromoplast fibrils equivalent to 500 μg of protein and containing 300 μg of total carotenoids was mixed for 5 min with hexane, diethyl ether, chloroform, or acetone (500 μL final volume) and incubated at 25°C for 10 min. The resulting homogenate was centrifuged in an Eppendorf microcentrifuge, and the organic phase was dried and dissolved in benzene to determine its absorbance at 480 nm. In the case of acetone, the lipid components were first partitioned into hexane before drying and transferring into benzene. For trypsin treatments, the fibril suspension was incubated at 25°C with 50 μg of trypsin (type III, Sigma) in the presence or absence of 100 μg of trypsin inhibitor (type III, Sigma). Likewise, phospholipase A2 (100 μg from Crotalus adamanteus, Sigma) or phospholipase C (25 μg from Clostridium perfringens, Sigma) was incubated with isolated fibrils prior to solvent extraction according to manufacturer's instructions.

In Vitro Fibril Reconstitution

Quantities of purified monogalactosyldiglyceride, digalactosyldiglyceride, and total phospholipids from chromoplast fibrils were dissolved in 25 μL of acetone before adding total carotenoids (in 25 μL of acetone) purified from isolated chromoplast fibrils. The resulting acetone solution was then added directly to a specified amount of purified fibrillin dissolved in 500 μL of 50 mM Tris buffer, pH 7.6. After 30 min at 25°C, the resulting mixture was subjected to three freeze cycles of 2 hr at −20°C, followed in each case by a thawing period of 30 min at 25°C and evaporation of the acetone. Following this treatment, 500 μL of diethyl ether was added to monitor the extent of reconstitution by measuring the decrease in carotenoid absorbance in the ether phase. For convenience, the ether phase was first dried under nitrogen before dissolving in benzene and measuring absorbance at 480 nm.

SDS-PAGE and Immunoblotting

Electrophoretic and immunoblotting techniques were performed as described previously by Kuntz et al. (1992). Antibodies raised against fibrillin according to standard procedures were used at a 1:2000 dilution.

Microscopy and Immunohistochemistry

Samples were prepared for electron microscopy as described previously (Camara and Brangeon, 1981), except for the silver proteinate staining, which was performed according to the method of Thiéry (1967). Indirect immunofluorescence staining was performed as described previously by Camara (1993), and the procedure of Tablin and Castro (1991) was adopted for immunogold labeling.

Analytical Techniques

Galactolipids and phospholipids were separated and determined as described previously (Camara and Monbger, 1977; Camara et al., 1983). Carotenoids and tocopherols were analyzed according to the methods of Camara and Monbger (1978), Camara et al. (1983), and Camara (1985a). Proteins were extracted as described by Meyer et al. (1988). Protein determinations were made with a protein assay kit (Bio-Rad).
ACKNOWLEDGMENTS

We wish to thank Drs. Alain Palloix and John R. Stommel for the generous gift of pepper seeds and useful discussions. We also thank Philippe Mirc for raising the antibodies. Dr. Jacques Schäfer for help during part of the microscopy work, and Annelyse Klein for expert technical assistance. S.R. was the recipient of a long-term European Molecular Biology Organization fellowship (No. ALTF 191-1990).

Received September 20, 1993; accepted November 23, 1993.

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*Plant Cell* 1994;6:119-133

DOI 10.1105/tpc.6.1.119

This information is current as of January 15, 2018