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A Ubiquitous Plant Housekeeping Gene, *PAP*, Encodes a Major Protein Component of Bell Pepper Chromoplasts[1]

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We have isolated a cDNA (*PAP*) corresponding to a single nuclear gene that encodes an approximately 30-kD major protein of bell pepper (*Capsicum annuum* L.) fruit chromoplasts. RNA and protein analyses revealed that, although at a low level, this gene is also expressed in every organ of the plant, the amount of the corresponding transcript and protein dramatically increasing in the latter stages of fruit development. Western-blot and immunocytochemical analyses of purified chloroplasts from leaves and fruits and of chromoplasts from red fruits showed that the encoded protein is the major component of plastoglobules and fibrils and is localized on the outer surface of these lipid structures. Analyses of *PAP* in plants belonging to different taxa revealed that it is expressed and highly conserved in both monocotyledonous and dicotyledonous plants. The presence of the protein in plastids not differentiating into chromoplasts indicates that *PAP* is expressed irrespective of the ontogeny of various plastid lines. In light of our results and since the encoded protein, identical to that previously named ChrB or fibrillin, is present in plastoglobules from several species and accumulates in the fibrils of bell pepper chromoplast, we propose to designate it as a plastid-lipid-associated protein.

Ripening is a highly regulated developmental process that takes place in fruits after seed maturation has been completed. It involves shifts in the metabolism upon the induction and stimulation of a large number of genes (Gillaspy et al., 1993). In many fruits chloroplasts differentiate into nonphotosynthetic chromoplasts with a concomitant degradation of chlorophylls and starch and extensive structural reorganization (Camara et al., 1989). Carotenoid accumulation involves the formation of distinct substructures that allows one to distinguish several classes of chromoplasts, the structural organization of which is well documented (Sitte et al., 1980).

Although during the past decade major advances have been made in understanding the factors governing the biogenesis of chloroplasts, there are limited data concerning the molecular aspects involved in the chloroplast to chromoplast transition during fruit ripening (Marano and Carrillo, 1992) and the role of plastid components in the acquisition of these specialized metabolic functions. A first step in the understanding of chromoplast biogenesis was the finding in bell pepper (*Capsicum annuum* L.) fruits of a carotenoid-binding protein designated ChrA (Newman et al., 1989; Cervantes-Cervantes et al., 1990), which is probably involved in the metabolism of carotenoids (Oren-Shamir et al., 1993; Bouvier et al., 1994; Houlné et al., 1994). Two major approximately 30-kD chromoplast-specific proteins have also been characterized in bell pepper: ChrB (Newman et al., 1989) and fibrillin (Deruère et al., 1994a, 1994b). The apparent absence of ChrB in extracts from unripe cv Albino fruits (Newman et al., 1989) and of fibrillin in extracts from unripe cvs Alma, Sweet Orange, and Jaune de Pignerolle (Deruère et al., 1994b) further confirms that these proteins are synthesized as part of the program of chromoplast development. Another chromoplast-specific 35-kD protein named ChrC has been characterized in the petals of cucumber (*Cucumis sativus* L.; Smirra et al., 1993). Recently, the cDNA corresponding to ChrC (Vishnevetsky et al., 1996) has been isolated and characterized; the predicted amino acid sequence shares significant homology with fibrillin.

With the aim of finding new and peculiar factors modulating the expression of genes involved in the development and ripening of the bell pepper fruit, we have chosen a ripening-specific cDNA obtained by differential screening as a tool. In fruits this cDNA encodes a plastid-associated protein, the level of which dramatically increases during the chloroplast to chromoplast transition. Using this cDNA as a probe, we were able to identify the corresponding gene and to study its expression pattern in different bell pepper tissues, as well as in several cultivars. The subcellular localization of the protein has been studied by both immunochemical and immunocytochemical methods. In addition, the presence of corresponding transcripts has been demonstrated in several plant species.

All of these data suggest that the protein is more widespread than expected from the studies of bell pepper ripening and that it may play an important role in the storage of lipophilic compounds in plastids of higher plants.

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Abbreviations: PAP, plastid-lipid-associated protein; RT, reverse transcriptase.

MATERIALS AND METHODS

Bell pepper (*Capsicum annuum*, Solanaceae, cvs Yolo Wonder, Albino, Golden Summer, and Permagreen), tomato (*Lycopersicon esculentum*, Solanaceae, cv Ailsa Craig), tobacco (*Nicotiana tabacum*, Solanaceae, cv SR1), barley (*Hordeum vulgare*, Gramineae, cv Himalaya), spinach (*Spinacia oleracea*, Chenopodiaceae, cv Monstrueux de Viroflay), and *Arabidopsis thaliana* (Brassicaceae, cv Columbia) were grown under greenhouse conditions.

Isolation of a Fruit-Ripening-Induced cDNA

A λgt11 cDNA library of a ripening cv Yolo Wonder fruit was differentially screened with labeled first-strand cDNA synthesized from poly(A^+) RNA of ripe fruits and leaves. A fruit-specific cDNA of 1210 bp, named *PAP*, was isolated and cloned into the *Eco*RI site of pBluescript KS^+ (Stratagene). DNA sequence and Southern-blot analyses were performed as previously described (Pozueta-Romero et al., 1995).

RNA Analysis

Total RNA from leaves, roots, flowers, and fruits of different plants was extracted and analyzed as described elsewhere (Pozueta-Romero et al., 1995). RT-PCR analyses of total DNase-treated RNA extracts were performed as described previously (Pozueta-Romero et al., 1996). For the synthesis of cDNA, the RNA was incubated 2 h at 37°C in 20 μL of RT assay mixture containing 5 μM oligonucleotide O_1 (5′-CCCAAGAATTCGACGTTCTCAGGC-3′, nucleotide position 754–731 of *PAP*). For amplification of the cDNA, 5 μL of the RT assay mixture was added to 50 μL of a PCR cocktail (Pozueta-Romero et al., 1996) containing either 1 μM each of O_1 and O_2 (5′-ATGGCTTCCATCTC TTCTCTCAATC-3′, nucleotide position 21–45 of *PAP*) (for the Solanaceae species) or 1 μM each of O1 and O3 (5′-GCCACAAATTACGACAAGGAAGATGAGTGG-3′, nucleotide position 199-to 227 of *PAP*) (for Arabidopsis and barley). Using a programmable thermal cycler (Prolabo, Paris, France) we performed amplification for 30 cycles, which consisted of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min.

Genomic DNA Analysis

Construction and Screening of the Genomic Library

Genomic DNA of cv Yolo Wonder was isolated, purified, ligated into λGEM-11 arms (Promega), and screened with the ^{32}P-labeled *PAP* cDNA as described elsewhere (Pozueta-Romero et al., 1995).

Plant Genomic DNA Amplification

DNA was isolated from leaves, according to the method of Bingham et al. (1981). DNA (0.5–1 μg) was amplified using the same set of gene-specific primers and with the same cycling protocol as described for RNA analysis.

Chloroplast and Chromoplast Fractionation

Chloroplasts were purified from 100 g of leaves or 100 g of mature green pepper fruits as previously described (Houlné et al., 1994). Chromoplasts from 200 g of fully ripe tomato fruits were purified as previously described (Hunt et al., 1986). Isolation of plastoglobules was performed basically as previously described (Bailey and Whyborn, 1963). The plastoglobules were released from lamellae and thylakoids of chloroplasts by short periods of ultrasonic treatment as previously described (Greenwood et al., 1963). The sonicated extract was centrifuged at 100,000*g* for 30 min on an SW-41 rotor (Beckman). The supernatant was subsequently centrifuged at 100,000*g* for 17 h. The floating pad was purified by a further flotation by addition of 1 M Suc and centrifugation at 100,000*g* for 3 h.

Electron Microscopy of Plastoglobules

The plastoglobule fraction obtained after centrifugation was either negatively stained with 1% uranyl acetate and observed on an H600 transmission electron microscope (Hitachi, Danbury, CT) or plated on a glass slide, air-dried, covered with 20 nm of AuPd, and examined with a Stereoscan 100 scanning electron microscope (Cambridge Instruments, UK).

Immunochemical Techniques

The *PAP* cDNA was subcloned into the *Eco*RI site of the pGEX-KG (Pharmacia) expression vector in frame to produce the protein as a glutathione *S*-transferase fusion protein. Induction of the fusion protein in *Escherichia coli* and production of specific antibodies were performed as described previously (Pozueta-Romero et al., 1995). Proteins from different plant tissues or from different plastid fractions were separated on SDS-PAGE and transferred to nitrocellulose filters for western-blot analysis.

Immunocytochemistry

Bell pepper plastids were isolated from fruits at various developmental stages as described above, but the 3500*g* pellets (containing plastids) were processed without further purification, either by chemical fixation or by cryofixation. Spinach chloroplasts were purified from leaves according to the method of Douce and Joyard (1979).

Chemical fixation was performed by progressively adding 20% paraformaldehyde and 25% glutaraldehyde in the extraction medium until final concentrations of 4% paraformaldehyde and 0.2% glutaraldehyde were reached. After 3 h at room temperature the plastids were pelleted and rinsed with extraction medium. The pellets were embedded in 1.5% agarose (low-temperature gelling) and dehydrated with ethanol with progressive lowering of temperature (Carlemalm et al., 1982). After infiltration with Lowicryl K4M at −20°C for 24 h the samples were embed-

ded in the resin and processed as described by Cheniclet and Rafia (1993).

Cryofixation of the plastid pellets was performed on a copper block cooled by liquid nitrogen, using the device developed by Verna (1983). The samples were freeze-substituted in acetone with 0.5% uranyl acetate added for 3 d at −87°C followed by 5 h at −20°C. The samples were infiltrated and embedded in Lowicryl K4M as indicated above. Spinach plastids were infiltrated with Suc after aldehydic fixation, fast-frozen, freeze-substituted, and embedded in Lowicryl K4M, according to the method of Rafia (1995).

The sections (70–90 nm thick) collected on naked 600-mesh or Parlodion-coated 200-mesh nickel grids were incubated according to the method of Cheniclet et al. (1992) and treated for 45 min with rabbit antibodies against the PAP protein diluted 1/1000 to 1/4000 in TBS-BSA. Goat anti-rabbit IgG conjugated with 10-nm gold particles (Biocell, Cardiff, UK) was used for the secondary labeling (45 min). The sections were then stained for 4 min with 2% aqueous uranyl acetate and for 30 s with 1% lead citrate at 20°C.

RESULTS

Identification of a Bell Pepper Housekeeping Gene, the Expression of Which Increases during Fruit Ripening

Northern-blot analysis using *PAP1* cDNA (accession no. X97118) as a probe detected a major band of about 1.2 kb (a size corresponding to that of the cDNA) and a minor band of approximately 2.2 kb. By rescreening we could isolate a cDNA identical to *PAP1* and containing a noncoding insertion of 814 bp likely corresponding to an intron (*PAP2*, accession no. X97559). It is interesting that the relative steady-state levels of the two different transcripts corresponding to pre-mRNA and to mature RNA vary considerably.

As shown in Figure 1A, the relative amount of the transcripts increases dramatically during the late stages of fruit development, whereas the transcripts are barely detectable in flowers and are not detectable, even after overexposure, in leaves. However, if the *PAP* mRNAs were only moderately expressed, then they might not be detected by RNA gel-blot analysis. Therefore, the transcript levels were further examined by a more sensitive RNA analysis using RT-PCR approaches that allowed us to detect *PAP* transcripts in leaves and roots (Fig. 1B). Hybridization signals were observed with two fragments of approximately 800 and 1600 bp, corresponding to the size of the mature mRNA and to the pre-mRNA, with PCR products amplified from cDNA prepared with total RNA leaf extracts. A transcript is also detected in cDNA from root RNA, but only the mature mRNA form is revealed by hybridization. Although the fragment obtained by PCR amplification of the cDNAs is clearly visible by staining, a hybridization with *PAP* cDNA as a probe was realized to ensure specificity. Taking into account the higher sensitivity of the RT-PCR method, one can assume that in leaves and roots the *PAP* transcripts are present at a 50- to 100-fold lower level. Thus, these results indicate that the corresponding gene is ubiquitously expressed in the plant, although its transcripts accumulate strongly in fruits during ripening.

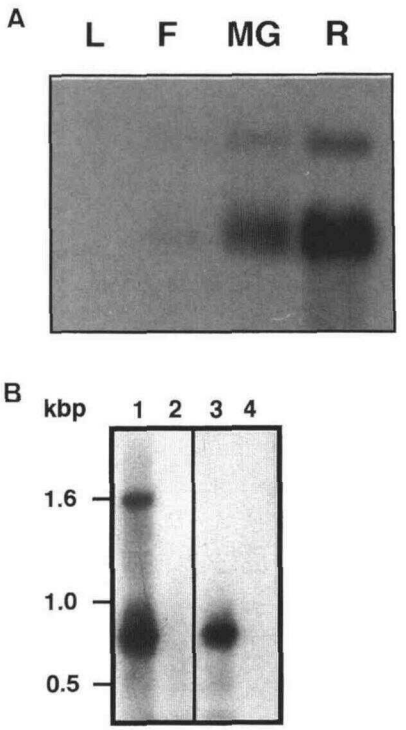

Figure 1. Expression of *PAP*. A, Northern analysis of the *PAP* transcripts. Ten micrograms of total RNA isolated from leaves (L), flowers (F), mature-green fruit (MG), and red-ripe fruit (R) were separated on agarose gels and hybridized with the radiolabeled *PAP* cDNA. B, RT-PCR analysis of total RNA extracts from leaves and roots using oligonucleotides O_1 and O_2 of the *PAP* cDNA as primers (see "Materials and Methods"). Amplification products were blotted and the filters hybridized with the radiolabeled *PAP* cDNA. Lane 1, PCR on leaf cDNAs; lane 2, PCR on total RNA of leaves; lane 3, PCR on root cDNAs; lane 4, PCR on total RNA of roots.

A database search revealed that the *PAP* cDNA has a striking identity with the sequence of a cDNA encoding a protein named fibrillin. Fibrillin has been described as a major chromoplast-specific protein of approximately 30 kD, the function of which is to sequester large amounts of carotenoids into fibril structures (Deruère et al., 1994b). The amino acid sequence analysis also showed that PAP is homologous to ChrB, a 30-kD major chromoplast-specific protein of bell pepper (Newman et al., 1989).

The sequence identities among PAP, fibrillin, and ChrB and the different expression patterns (Deruère et al., 1994b; this work) raise the question of the number of the genes encoding these proteins. To this end, cv Yolo Wonder genomic DNA was examined by Southern-blot hybridization. As shown in Figure 2, the number of DNA fragments detected and their size correlate with the restriction pattern of the two types of cDNAs (*PAP1* and *PAP2*). This hybridization pattern and the fact that only one family of genomic clones has been isolated suggest that *PAP* is monogenic, thus confirming that ChrB, fibrillin, and PAP represent the same protein encoded by a single gene.

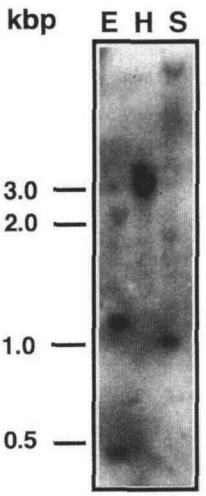

Figure 2. Structure of the *PAP* gene. Southern hybridization of 10 μg of genomic DNA restricted with *EcoRI* (E), *StyI* (S), and *HindIII* (H). The blot was probed with ^{32}P-labeled *PAP* cDNA.

Expression Pattern of PAP in Various Strains and Cultivars of Bell Pepper

In our first attempt to examine whether the expressions at the protein and at the transcript level are similar, we performed western-blot analysis using antisera raised against the protein overexpressed in *E. coli*. As shown in Figure 3A, PAP is detected in leaves, flowers, and fruits. The relative amount of protein is about 20 times higher in fruit than in leaves and increases dramatically during fruit ripening, from the mature-green to the red stage.

Second, since fibrillin was shown to occur only in chromoplasts and only in the presence of cyclic carotenoids (Deruère et al., 1994b), we addressed the problem of the presence or absence of PAP in other bell pepper cultivars characterized by their plastidial structures. In cv Albino, ripening corresponds to a color development from white to red. On a structural basis instead of chloroplasts, the unripe fruits contain proplastids, which develop into chromoplasts (Kirk, 1967). Golden summer is a cultivar in which the mature-green fruit develops a deep-yellow color at the final ripening stage, as a consequence of an impaired carotenoid biosynthetic pathway (Kirk, 1967; Simpson et al., 1974). The fruit of cv Permagreen are green throughout fruit development and are devoid of fibril-like structures. During ripening they only accumulate the chloroplast type of carotenoids (Oren-Shamir et al., 1993).

As can be observed in Figure 3B, the ripening of fruits of cvs Albino and Golden Summer is characterized by a dramatic increase of levels of PAP already existing in the unripe white and green fruits, respectively. Unripe green fruits of cv Permagreen contain normal levels of PAP, as compared with the other cultivars analyzed. During fruit development the amount of *PAP* remains almost constant. Thus, the pattern of PAP protein modulation parallels that of the *PAP* transcript level. Altogether these results confirm that *PAP* is a gene that, although expressed in all plant tissues, is developmentally controlled and mainly expressed in fruits at the latter stages of ripening.

Subcellular Localization of PAP

The above results show that *PAP* is a ubiquitously expressed gene, the encoded protein of which is identical to fibrillin, which has been shown to be the major protein component of the bell pepper fibrils (Deruère et al., 1994b). Fibrils are exclusively present in the chromoplasts of normal, red-ripe fruits. Thus, two questions arise: First, since fibrils are chromoplast-specific structures, where is PAP localized in organs not containing chromoplasts, such as leaves and green fruits, or in plastids of roots in which the gene is also expressed (Fig. 1)? Second, in fibril-containing chromoplasts, is PAP exclusively localized in these structures? To answer these questions, we have performed western-blot and immunocytochemical analyses. Protein extracts from chloroplasts purified from leaves and from green fruits were analyzed. In these fractions antisera raised against PAP detected a protein band of approximately 30 kD. Further fractionation of the purified chloroplasts allowed us to localize more precisely PAP within the chloroplast of leaves and green fruits (Fig. 4A). The protein was detected in both the membrane and soluble fractions. However, the strongest signal was obtained with the 100,000*g* supernatant fraction of lysed chloroplasts.

The hypothesis that, during the chloroplast to chromoplast transition, fibrils arise from plastoglobules (Simpson et al., 1974; Knoth et al., 1986) prompted us to analyze the

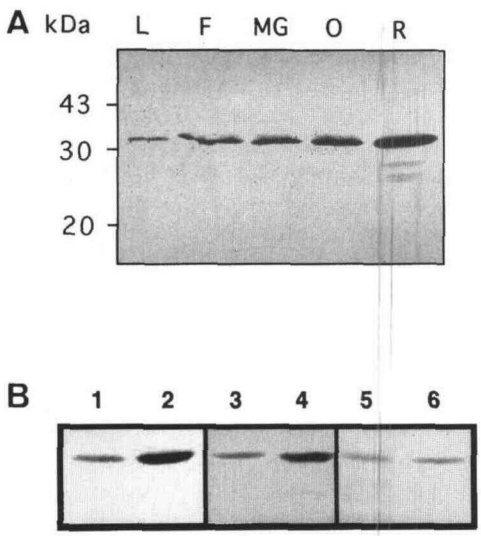

Figure 3. A, Immunological analysis of PAP in samples from leaves (L), flowers (F), and mature-green (MG), orange (O), and red-ripe (R) fruits. For leaves and flowers, and for fruits, 40 and 10 μg of protein, respectively, were subjected to 12% SDS-PAGE, followed by immunoblotting using antisera raised against the overexpressed protein encoded by the *PAP* cDNA. B, Western blot of PAP on total protein extracts from fruits of three cultivars of bell pepper at different developmental stages. In each case, 10 μg of protein was subjected to 12% SDS-PAGE. Lane 1, Unripe fruit of cv Albino; lane 2, red fruit of cv Albino (approximately 4–5 weeks after anthesis); lane 3, unripe fruit of cv Golden Summer; lane 4, ripe yellow fruit of cv Golden Summer (approximately 4–5 weeks after anthesis); lane 5, unripe fruit of cv Permagreen; lane 6, fruit of cv Permagreen 4–5 weeks after anthesis.

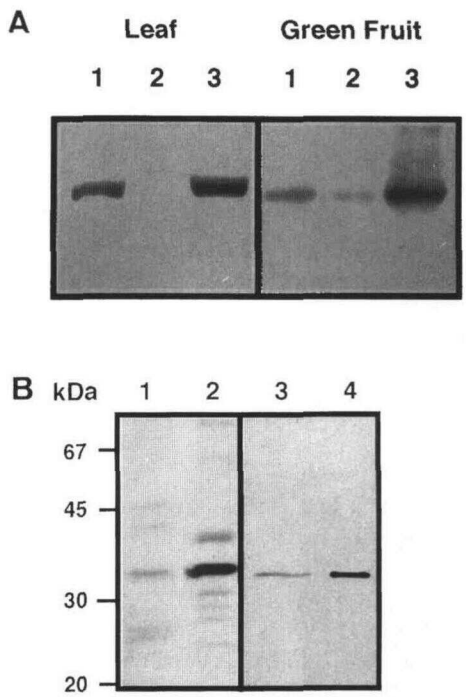

Figure 4. Isolation and immunodetection of PAP in the chloroplast of leaves and of unripe bell pepper fruits. A, Immunochemical detection of PAP in total (lane 1), membrane (lane 2), and stromal (lane 3) fractions of fruit and leaf chloroplasts. B, Silver stain of proteins from plastoglobules isolated from the stromal fraction of leaf and green fruit chloroplasts separated on 12% SDS-PAGE (lanes 1 and 2, respectively), and immunodetection of PAP in plastoglobule proteins isolated from leaf and green fruit chloroplasts (lanes 3 and 4, respectively).

latter type of lipoprotein structures. By using the 100,000*g* supernatant as a starting material, following the procedure described in "Materials and Methods," we could obtain a yellow fraction enriched in plastoglobules. As shown in Figure 4B, a major 30-kD protein is revealed by staining and is specifically recognized by the PAP antibodies. Electron-microscopy analysis (Fig. 5) clearly indicates that the fraction contained mainly plastoglobules identical to those observed in vivo and showed negligible contamination with other plastid substructures.

In parallel, PAP could be localized by immunocytochemical methods on plastids isolated from bell pepper fruits at different ripening stages (Fig. 6). During ripening the thylakoids present in the chloroplasts of green fruits progressively disappear and, instead, fibrils, tubules, and peripheral membranes develop, giving rise to characteristic fibrillar chromoplasts (Rafia, 1995).

In the first attempt immunocytochemistry was performed on bell pepper fruit samples that were processed according to various experimental procedures (see "Materials and Methods"). However, no successful labeling with anti-PAP antibodies could be obtained, due to morphological specific features of bell pepper fruit tissue. Therefore, this situation prompted us to use isolated plastids that are more suitable for the immunolocalization of most plastid antigens (Cheniclet et al., 1992; C. Cheniclet, unpublished data). The procedure used yields a population containing a majority of intact plastids, as illustrated particularly by the high density of the stroma and the presence of the plastid envelope (which is only faintly contrasted in the absence of osmium tetroxide as a fixative).

In chromoplasts the density of gold particles is very high (Fig. 6, A and B), whereas contaminating mitochondria are not labeled (data not shown). The immunolabeling is concentrated mainly in the plastid areas where fibrils are present, whereas the tubule areas are only weakly labeled. The gold particles are not scattered at random over fibril transections but are closely associated with their boundary layer (Fig. 6B). A similar observation is made on the bundles of tubules, which are visible either as longitudinal trans-sections or as cross-sections. This localization of the fibril immunolabeling is consistent with the model proposed by Knoth et al. (1986), in which carotenoids accumulate in the center and are surrounded by a layer of protein.

Immunolabeling with PAP has been followed in plastids at different ripening stages of the bell pepper fruits. In chloroplasts from green fruits (Fig. 6C), in which typical thylakoids are present and fibrils have not yet developed, a discrete labeling is observed, confirming the presence of PAP at this stage (Fig. 3A). In these plastids the gold particles are present only over the plastoglobule areas. This observation correlates with the detection of PAP in the plastoglobule-enriched fraction (Fig. 4B). As for fibrils, the gold particles are not located inside the globules but on the boundary layer (Fig. 6D), indicating that PAP is part of the interface between the stroma and the lipophilic core of globules. This corresponds to the model of plastoglobule structure proposed by Hansmann and Sitte (1982).

During the first steps of fruit ripening, the labeling intensity of plastids increases, more by plastoglobule multiplication than by enlargement of individual plastoglobules. In chloro-chromoplasts from intermediate ripening stages (Fig. 6, E and F), in which thylakoids coexist with fibrils elongating from plastoglobules, the PAP labeling is far more intense on the globulo-fibrillar structures than over the other plastid compartments. In light of these results the PAP protein cannot be considered only as a fibril-specific protein. It appears rather as a plastoglobule-associated protein, which accumulates in the fibrils during bell pepper chromoplast differentiation. Therefore, we propose to designate it as a plastidial, lipid-associated protein.

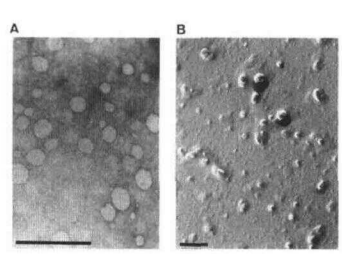

Figure 5. Electron micrographs of isolated and purified plastoglobules from bell pepper fruits. A, Negative staining. Bar represents 1 μm. B, Scanning-electron micrograph of plastoglobules as in A. Bar represents 10 μm.

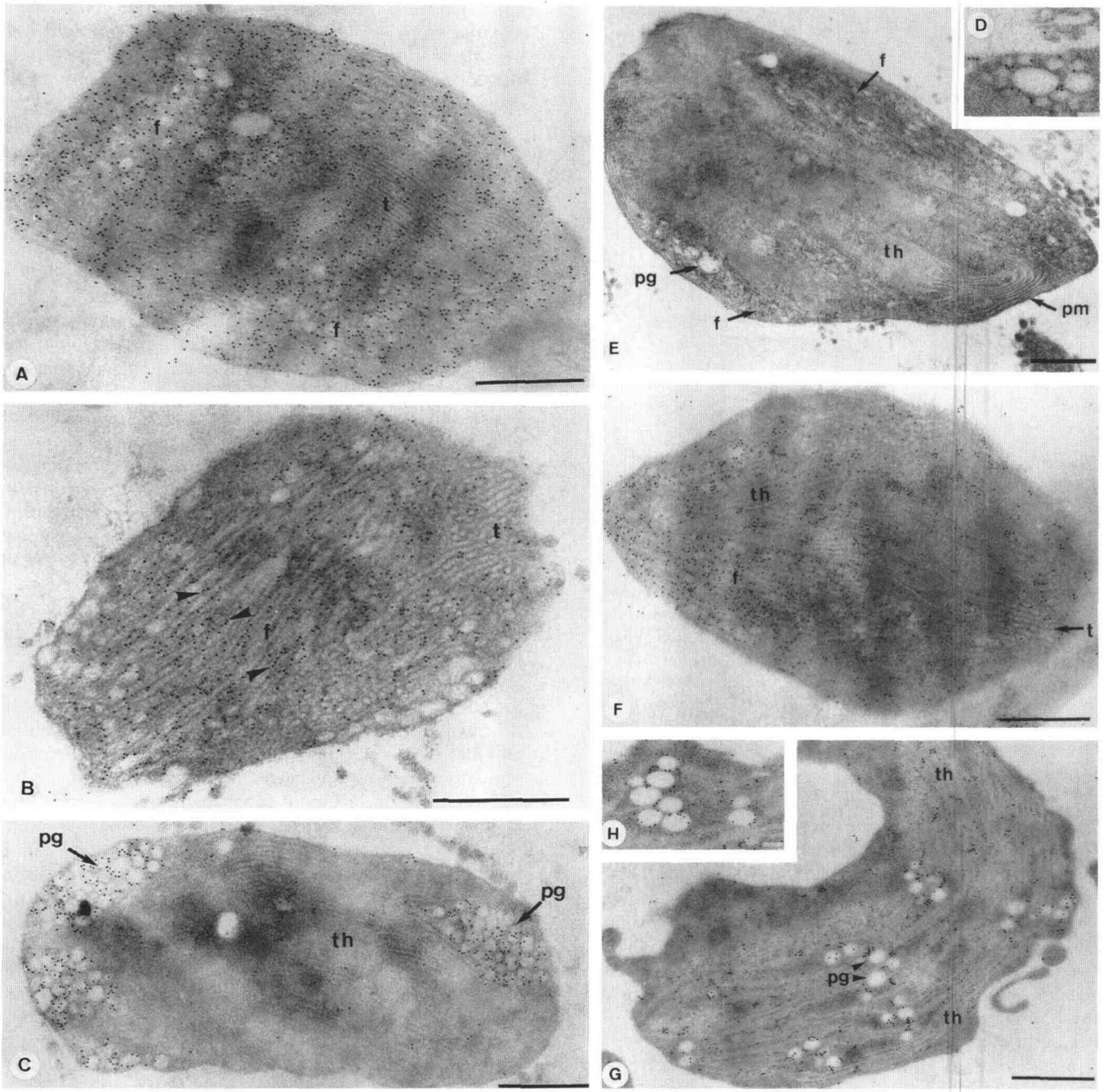

Figure 6. Immunogold localization of PAP in isolated plastids, secondary antibodies are conjugated to 10-nm gold particles. A and B, Chromoplasts from ripe bell pepper fruits (cryofixation). C, D, and E, Plastids from bell pepper fruits at the early ripening stages (cryofixation). F, Chloro-chromoplasts from bell pepper fruit (late-transition stage, cryofixation). G and H, Spinach chloroplasts (aldehyde fixation followed by cryofixation). Scale bars: D and H, 0.1 μm; A to C and E to G, 0.5 μm. t, Tubule areas; f, fibril; pg, plastoglobules; pm, peripheral membranes; th, thylakoids.

Occurrence and Localization of PAP in Other Species

The presence of plastoglobules has been described in all plastid types of many plants (Bailey et al., 1967) and, therefore, it was very likely that PAP may exist in species other than bell pepper. To determine the occurrence of PAP in various plants we tested a PCR amplification of genomic DNA from different dicotyledonous plants, tomato, tobacco, and Arabidopsis, and the monocotyledonous plant barley, using *PAP*-specific oligonucleotides as primers. By using O_1 and O_3 (see "Materials and Methods") a specific fragment (hybridizing with the bell pepper cDNA) could be obtained with all of the genomic DNAs tested (data not shown). It is interesting that with the DNA of different Solanaceae (tomato and tobacco), even the pair of oligonu-

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            1                                                   50
    Pepper  MASISSLNQI PCKTLQITSQ YSKISSLPLT SPNFPSKTEL HRSISIKEFT
      ChrC  MAFVSQFNQL PCKTLALNPP QPQLTSKPSV FPIASIGAT. ARAAAGKSLI
 Tobacco1                §IQQI TSHYSKPTSK ISTFPITSTN FPSISVKEFT
  Tomato1       §IPWQT LQITSQYSKP TSKISTLPIS STNFPSKTEL HRAISVKEFT

            51                                                  100
    Pepper  NPKPKFTAQA TNYDKEDEWG PELEQINP.. GGVAVVEEEP PKEPSEMEKL
      ChrC  SVRPAFKVRA VLNDDEWGED KDEKYGDDSS VAVAEKEEEK PLEPSEIYKL
 Tobacco1  NPKPKFTAQA KNYDKDDEWG PEVEQIKPSG GGVSVAEEEP PKEPSEIELL
  Tomato1  YPKPKFTAQA TNYDKEDEWG PEVEKISP.. GGVAVVDEEP PKEPSEIELL
    Barley                        §EQINP.. GGVAVVEEEP PKEPSEMEKL
  Tomato2           § TNYDKEDEWG PEVEKLSP.. GGVAVVDEEP PKEPSEIELL
  Potato1         §A TNYDKEDEWG PEVEQI§

            101                                                 150
    Pepper  KKQLTDSFYG TNRGLSASSE TRAEIVELIT QLESKNPTPA PTEALSLLNG
      ChrC  KKALVDSFYG TDRGLRVSRD TRAEIVELIT QLESKNPTPA PTEALTLLNG
 Tobacco1  KKQLVDSFYG TNRGCSASSE TRAEIVELIT KLESMNPTPA PTEALPLLNG
  Tomato1  KKQLADSFYG TNRGLSASSE TRAEIVELIT QLESKNPNPA PTEALTLLNG
    Barley  KKQLTDSFYG TNRGLSATSE SWAEIVELIT QLESKNPTPA PTEALSLLNG
  Tomato2  KKQLADSFYG TNRGLSASSE TRAEIVELIT QLESKNPNPA PTEALTLLNG

            151                                                 200
    Pepper  KWILAYTSFS GLFPLLARGN LLPVRVEEIS QTIDAETLTV QNSVVFAGPL
      ChrC  KWILAYTTFA GLFPLLSR.N LPLVKVEEIS QTIDSENLTV QNSVQFSGPL
 Tobacco1  KWILAYTSFS GLFPLLSRGT LPLVRVEEIS QTIDSETFAV QNCVVFAGPL
  Tomato1  KWILAYTSFS GLFPLLSRGN LLLVRVEEIS QTIDSESFTV QNSVVFAGPL
    Barley  KWILAYTSFS GLFPLLARGN LLPVRVEEIS QTIDAETLTV QNSVVFAGPL
  Tomato2  KWILAYTSFS GLFPSLSRGN LLLVRVEEIS QTIDSESFTV QDSAVFAGPL
 Tobacco2                                                §VVNAGPI

            201                                                 250
    Pepper  STTSISTNAK FEVRSPKRLQ INFEEGIIGT PQLTDSIELP ENVEFLGQKI
      ChrC  ATTSITTNAK FEVRSPLRVH IKFEEGVIGT PQLTDSIVIP DNVDFLGQKI
 Tobacco1  ATTSITTNAK FEVRSPKRVQ IKFDEGIIGT PQLTD§
  Tomato1  ATTSISTNAK FEVRSPKRVQ IKFEEGIIGT PQLTD§
    Barley  STTSISTNAK FEVRSPKRLQ INFEEGIIGT PQLTDSI§
Arabidopsis §TTSISTNAK FEVRSPKRLQ INFVEGGVGT PQLTESI§
  Tomato2  STTSIST§
 Tobacco2  GTTSITTNAK FEVRSPKRVQ IKFDEGVIGT PQLT§

            251                                                 300
    Pepper  DLSPFKGLIT SVQDTATSVA .KSISSQPPI KFPISN SYA QSWLLTTYLD
      ChrC  DFTPFNGIIS SLQDTASNVA .KTISSQPPI KFSISNT.RV ESWLLTTYLD
  Potato2                        §SISSQPPI KFPIINNNAA Q.EHSNPY§

            301                 326
    Pepper  AELRISRGDA GSIFVLIKEG SPLLKP
      ChrC  EDLRISRGDG GSVFVLLKEG SSFLSL
```

Figure 7. Presence and expression of *PAP* in different plant species. Comparison of amino acid sequences predicted from the PCR genomic fragments and RT-PCR fragments obtained using *PAP*-specific oligonucleotides as primers (see "Materials and Methods"). Tomato 1 and tobacco 1 are sequences deduced from RT-PCR experiments. Arabidopsis, tomato 2, and tobacco 2 are sequences deduced from PCR performed on genomic DNA. Potato 1 and potato 2 concern two peptide sequences obtained by protein sequencing (Pruvot et al., 1996). ChrC is from Vishnevetsky et al. (1996). § indicates the ends of determined sequences.

cleotides O_2 and O_1 generated specific fragments (data not shown), indicating that the sequence encoding the transit peptide is highly conserved.

To test whether the *PAP* genes from different species are expressed, a series of experiments were performed in which RT-PCR amplification from leaf RNA was used. Partial sequencing of the different fragments obtained by PCR on genomic DNA or by RT-PCR allowed the comparison of the deduced amino acid sequences shown in Figure 7. We have included in the figure data concerning two peptide sequence determinations from a potato drought-induced protein published by Pruvot et al. (1996), as well as the recently published protein of the other chromoplast-specific protein from cucumber ChrC (Vishnevetsky et al., 1996). The comparison shown in Figure 7 indicates a very high homology among all of the species analyzed, ranging from 70 to 92%. Surprisingly, the first peptide of the potato drought-induced protein shares 96% homology with the bell pepper PAP. Even with a relative low homology found between the second peptide and PAP (52%), these results enabled us to identify the potato protein as PAP. In addition, confirming the conservation of the transit peptide among the Solanaceae, the deduced amino acid sequences from tomato and tobacco are 36 and 67%, respectively, homologous to the bell pepper counterpart.

To obtain further information concerning the suborganellar localization of PAP in other species, we performed a fractionation of chloroplasts and chromoplasts from tomato fruits. The fractionation of the purified chromoplasts, following the same procedure as that used for bell pepper, allowed the analysis of a sample highly enriched in plastoglobules. As shown in Figure 8, a major protein of approximately 30 kD is specifically recognized by the antiserum. A faint band could also be obtained in chloroplasts isolated from leaves or from green unripe fruits (data not shown) by overloading the gels.

Immunocytochemical observations using bell pepper anti-PAP antibodies were also performed on isolated chloroplasts purified from spinach leaves and processed as described in "Materials and Methods." Spinach plastids also contain many plastoglobules often arranged in clusters. A distinct and specific labeling was observed on plastid sections, concerning mainly the clusters of plastoglobules and, at a lower level, the thylakoid membranes (Fig. 6G). In addition, the gold particles were clearly localized at the interface between adjacent globules, as well as between the plastoglobules and the plastid stroma (Fig. 6H). These observations showed that a plastid protein from sources other than Solanaceae could be recognized by the bell pepper antisera and that it is localized at the same suborganellar level, the plastoglobules.

Altogether these results indicate that *PAP* is a widespread gene expressed in various higher plants. The encoded protein is present in both chlorophyllous and achlorophyllous plastids independently of their internal organization. The presence of PAP as a major protein of chloroplast plastoglobules and of chromoplast extracts confirms the hypothesis that plastoglobules and fibrils are

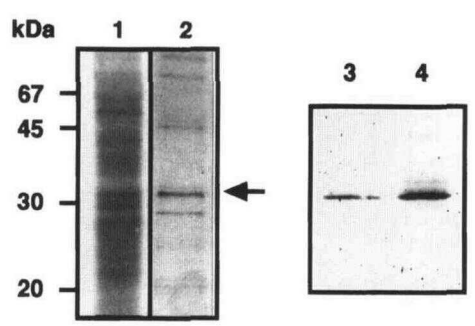

Figure 8. Suborganellar localization of PAP in tomato plastids. Silver stain of protein extracts from purified chromoplasts (lane 1) and plastoglobules (lane 2) (10 and 0.3 μg of protein, respectively, were loaded onto the gel) and the corresponding immunodetection of PAP in chromoplast extracts (lane 3), and plastoglobules (lane 4). The proteins were separated by 12% SDS-PAGE.

ontogenetically related in the course of plastid transformation concomitantly with carotenoid accumulation and shows that *PAP* expression precedes greatly fibril formation.

DISCUSSION

Ripening of the bell pepper fruit involves conversion of chloroplasts to chromoplasts, which is characterized by the accumulation of carotenoids and by the degradation of thylakoid membranes and chlorophylls. Carotenoids are normal constituents of the plastid membranes and they function as electron carriers. Their accumulation in chloroplasts is tightly regulated and closely coordinated with the assembly of the photosynthetic apparatus. On the other hand, abundant carotenoids in pigmented flowers and fruits are necessary to ensure successful reproductive development of the species. Carotenoids of plastids in large amounts may crystallize to form rigid, unevenly shaped bodies (e.g. lycopene in the crystalline-type chromoplast of tomato fruits, Harris and Spurr, 1969a). They are also sequestered into specialized lipoprotein elements, such as plastoglobules (Hansmann and Sitte, 1982) or fibrils (Knoth et al., 1986; Deruère et al., 1994b).

In the present work we have demonstrated that in bell pepper the proteins designated ChrB (Newman et al., 1989), fibrillin (Deruère et al., 1994b), and PAP (our work) are encoded by the same single gene and therefore represent the same protein.

This protein was named fibrillin by Deruère et al. (1994b), because it is the major protein component of chromoplast fibrils. Here we show that the protein is also abundant in tomato chromoplasts, where carotenoid accumulation does not involve the formation of typical fibrils (Harris and Spurr, 1969b), as well as in protein extracts of bell pepper cultivars (e.g. Albino and Golden Summer), which accumulate the protein in the course of ripening but do not contain fibrillar chromoplasts. In addition, although at a low level, this protein is also present in bell pepper chloroplasts from leaf and green fruits. Moreover, the gene is also expressed in tissues, such as roots, containing noncolored plastids. Finally, we observed that this protein can be immunologically recognized in spinach chloroplasts. Taken together, these results indicate that the fibrillin is not specific to bell pepper chromoplasts but is a protein that is associated with the plastid compartment of various plant species and accumulates during fruit ripening jointly with a very high carotenoid neosynthesis. By immunological methods, we have shown in tomato chromoplasts, as well as in bell pepper and spinach chloroplasts, that the protein is associated with plastoglobules.

Plastoglobules are common constituents of any plastid type and are localized within the plastid stroma (Greenwood et al., 1963). However, their size, number, and electronic density after osmium fixation are very variable. In chloroplasts the total volume of plastoglobules increases as the plastid expands and reaches a maximum in the leaves just before falling, whereas grana and stroma thylakoids become highly disorganized (Hudak, 1981; Hashimoto et al., 1989), or in green fruits at the end of the maturation stage (Camara et al., 1989). When chromoplasts are developing from chloroplasts, plastoglobules are either the unique compartment where carotenoids accumulate (e.g. *Viola tricolor*, Hansmann and Sitte, 1982) or subsidiary sites where crystals (tomato, Harris and Spurr, 1969b) or fibrils (bell pepper, Simpson et al., 1974) are conjointly developed.

The composition of purified plastoglobules from chromoplasts and chloroplasts has been determined in a few cases (Greenwood et al., 1963; Hansmann and Sitte, 1982; Young et al., 1991). In addition to triacylglycerols and to carotenoids or plastoquinones they always contain a small amount of polar lipids (4–7%) and proteins (3–5%). However, the presence of proteins as genuine components of plastid globules has been questioned, owing to the difficulty in obtaining pure plastoglobules and the loss of plastoglobules proteins after several purification steps (Steinmüller and Tevini, 1985).

The difference in lipid composition between chloroplast and chromoplast plastoglobules raises the question of the existence of two different lines of plastoglobules (Simpson and Lee, 1976).

The immunocytochemical observations presented in this work, concerning either bell pepper fruit chloroplasts at incipient fruit ripening or spinach chloroplasts, support the hypothesis that proteins are actually part of the plastoglobule structure and in addition are constitutive of their boundary layer, as for fibrils. We have shown that immunolabeling is not internalized but is located at the interface between the hydrophobic core and the surrounding hydrophilic stroma.

After osmium tetroxide fixation, the boundary layer of plastoglobules is more electron dense than the core, which could be related to the presence of a half-unit membrane, including polar lipids and proteins (Hansmann and Sitte, 1982). In this respect, the situation could be similar to that of oleosomes, which are cytoplasmic particles within specialized plant cells accumulating oil (Napier et al., 1996). Oil bodies, containing mostly triacylglycerols, are delineated from the surrounding cytosol by a layer of dense material, which has been shown to be a half-unit membrane (Murphy and Cummins, 1989). The main protein components of the oleosomes, oleosins, are integrated within this boundary layer (Huang, 1992).

The similar structural organization of plastoglobules and oleosomes could account for an equivalent role of PAP in plastids and oleosin in cytosol, i.e. building a compatible interface when large amounts of triacylglycerols, carotenoids, or quinones are stored in an aqueous phase. Such a role could explain the presence of PAP throughout the plastid developmental process.

The highly conserved amino acid sequence of PAP among the different plant species indicates that this protein plays an important role in the formation of structures that function as lipid-sequestering elements. Several reports of the presence of a 30-kD protein in fibrils or globules from different species than those analyzed in the present work, such as *Tropaeolum majus* L., *Cucurbita maxima* (Deruère et al., 1994b), *Rosa rugosa*, *Palisota barteri* (Knoth et al., 1986), or *V. tricolor* (Hansmann and Sitte, 1982), reinforce the hypothesis of the ubiquity of PAP. Similarly, *Thunbergia*

flowers contain a chromoplast-specific 32-kD protein (Ljubesic et al., 1996). Another chromoplast-specific protein of 35 kD named ChrC has been characterized in the petals of *C. sativus* (Smirra et al., 1993). The corresponding cDNA has been isolated and sequenced recently (Vishnevetsky et al., 1996). The homology shared by ChrC with PAP is very high, as expected, with the exception of the N-terminal end, which is completely different over the first 24 residues. Considering the similarity in function (carotenoid association), this divergence in the N-terminal part, highly conserved in the other species analyzed, would indicate that this region is likely specific for another function in cucumber.

However, the fact that a drought-induced protein from potato (Pruvot et al., 1996) could be identified as a marker of the PAP family may indicate that these proteins are also involved in other cellular functions in addition to passive lipid storage. Such other important functions could explain the high degree of protein sequence conservation during evolution. Analysis of the PAP sequence does not show obvious functional parts. Only the peptide RGD, known to be involved in cell adhesion (D'Souza et al., 1991), could be characterized. From the work of Pruvot et al. (1996), it appears that this protein not only plays a structural role in cell protection during dehydration but it is also likely involved in the hardening process of the chloroplast after a drought stress. The fact that this 34-kD protein is located at the interphase water-membrane in *Solanum* plastids further strengthens the hypothesis that PAP proteins could be associated with various situations involving a local decrease of water concentration.

Our results showing the presence of the *PAP* gene in different plant species open a very important and interesting field of investigation concerned with the structure of the gene and the regulation of its expression. Preliminary results obtained while analyzing our PCR products indicate that the structure is highly conserved in the Solanaceae species. An intron similar to that found in the nonspliced cDNA species is also present. However, the levels of pre-mRNA of *PAP* in bell pepper are abnormally high (Fig. 1A), whereas they are undetectable in the other species analyzed. In addition, the study of the regulation mechanisms controlling *PAP* expression in cells with different types of plastids and with plastids undergoing various types of differentiation represents an important aspect for the understanding of plastid ontogeny and is currently under way.

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LITERATURE CITED

Bailey JL, Thornber JP, Whyborne AG (1967) The chemical nature of chloroplast lamellae. *In* TW Goodwin, ed, Biochemistry of Chloroplasts, Ed 1. Academic Press, London, pp 243–256

Bailey JL, Whyborn AG (1963) The osmiophilic globules of chloroplasts. II. Globules of the spinach-beet chloroplast. Biochim Biophys Acta **78:** 163–174

Bingham PM, Levis R, Rubin GM (1981) Cloning of DNA sequences from the *white* locus of *D. melanogaster* by a novel and general method. Cell **25:** 693–704

Bouvier F, Hugueney P, d'Harlingue A, Kuntz M, Camara B (1994) Xanthophyll biosynthesis in chromoplasts: isolation and molecular cloning of an enzyme catalyzing the conversion of 5,6-epoxycarotenoid into ketocarotenoid. Plant J **6:** 45–54

Camara B, Bousquet J, Cheniclet C, Carde JP, Kuntz M, Evrard JL, Weil JH (1989) Enzymology of isoprenoid biosynthesis and expression of plastid and nuclear genes during chromoplast differentiation in pepper fruits (*Capsicum annuum*). *In* CD Boyer, JC Shannon, RC Hardison, eds, Biochemistry and Genetics of Nongreen Plastids. American Society of Plant Physiologists, Rockville, MD, pp 141–156

Carlemalm E, Garavito RM, Villiger W (1982) Resin development for electron microscopy and an analysis of embedding at low temperature. J Microsc **126:** 123–143

Cervantes-Cervantes M, Hadjeb M, Newman LA, Price CA (1990) ChrA is a carotenoid-binding protein in chromoplasts of *Capsicum annuum*. Plant Physiol **92:** 1241–1243

Cheniclet C, Rafia F (1993) Les résines acryliques pour l'immunocytochimie en microscopie electronique: intérêt de l'inclusion à basse température. Rev Fr Histotechnol **6:** 38–47

Cheniclet C, Rafia F, Saint-Guily A, Verna A, Carde J-P (1992) Localization of the enzyme geranylgeranylpyrophosphate synthase in *Capsicum* fruits by immunogold cytochemistry after conventional chemical fixation or quick-freezing followed by freeze substitution. Labelling evolution during fruit ripening. Biol Cell **75:** 145–154

Deruère J, Bouvier F, Steppuhn J, Klein A-L, Camara B, Kuntz M (1994a) Structure and expression of two plant genes encoding chromoplast-specific proteins: occurrence of partially spliced transcripts. Biochem Biophys Res Commun **199:** 1144–1150

Deruère J, Römer S, d'Harlingue A, Backhaus RA, Kuntz M, Camara B (1994b) Fibril assembly and carotenoid overaccumulation in chromoplasts: a model for supramolecular lipoprotein structures. Plant Cell **6:** 119–133

Douce R, Joyard J (1979) Structure and function on the plastid envelope. Adv Bot Res **7:** 1–116

D'Souza S, Ginsberg M, Plow E (1991) Arginyl-glycyl-aspartic acid (RGD) a cell adhesion motif. Trends Biol Sci **16:** 246–250

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell **5:** 1439–1451

Greenwood AD, Leech RM, Williams JP (1963) The osmiophilic globules of chloroplasts. I. Osmiophilic globules as a normal component of chloroplasts and their isolation and composition in *Vicia faba* L. Biochim Biophys Acta **78:** 148–162

Hansmann P, Sitte P (1982) Composition and molecular structure of chromoplast globules of *Viola tricolor*. Plant Cell Rep **1:** 111–114

Harris WM, Spurr AR (1969a) Chromoplasts of tomato fruits. I. Ultrastructure of low pigment and high-beta mutants. Carotene analysis. Am J Bot **56:** 369–379

Harris WM, Spurr AR (1969b) Chromoplasts of tomato fruits. II. The red tomato. Carotene analysis. Am J Bot **56:** 380–389

Hashimoto H, Kura-Hotta M, Kato S (1989) Changes in protein content and in the structure and number of chloroplasts during leaf senescence in rice seedlings. Plant Cell Physiol **30:** 707–715

Houlné G, Schantz ML, Meyer B, Pozueta-Romero J, Schantz R (1994) A chromoplast-specific protein in *Capsicum annuum*: characterization and expression of the corresponding gene. Curr Genet **26:** 524–527

Huang AHC (1992) Oil bodies and oleosins in seeds. Annu Rev Plant Physiol Plant Mol Biol **43:** 177–200

Hudak J (1981) Plastid senescence. 1. Changes of chloroplast structure during natural senescence in cotyledons of *Sinapis alba* L. Photosynthetica **15:** 174–181

Hunt CM, Hardison RC, Boyer CD (1986) Restriction enzyme analysis of tomato chloroplast and chromoplast DNA. Plant Physiol **82:** 1145–1147

Kirk JTO (1967) The chromoplast. *In* JTO Kirk, RAE Bassett Tilney, eds, The Plastids. Their Chemistry, Structure, Growth and Inheritance. WH Freeman, San Francisco, CA, pp 72–79

Knoth R, Hansmann P, Sitte P (1986) Chromoplasts of *Palisota barteri*, and the molecular structure of chromoplast tubules. Planta **168:** 167–174

Ljubesic N, Wrischer M, Devidé Z (1996) Chromoplast structures in *Thunbergia* flowers. Protoplasma **193:** 174–180

Marano MR, Carrillo N (1992) Constitutive transcription and stable RNA accumulation in plastids during the conversion of chloroplasts to chromoplasts in ripening tomato fruits. Plant Physiol **100:** 1103–1113

Murphy DJ, Cummins I (1989) Purification and immunogold localisation of the major oil body membrane protein of oilseed rape. Plant Sci **60:** 47–54

Napier JA, Stobart AK, Shewry PR (1996) The structure and biogenesis of plant oil bodies: the role of the ER membrane and the oleosin class of proteins. Plant Mol Biol **31:** 945–956

Newman LA, Hadjeb N, Price CA (1989) Synthesis of two chromoplast-specific proteins during fruit development in *Capsicum annuum*. Plant Physiol **91:** 455–458

Oren-Shamir M, Hadjeb N, Newman LA, Price CA (1993) Occurrence of the chromoplast protein ChrA correlates with a fruit-color gene in *Capsicum annuum*. Plant Mol Biol **21:** 549–554

Pozueta-Romero J, Houlné G, Schantz R (1996) Nonautonomous inverted repeat *Alien* transposable elements are associated with genes of both monocotyledonous and dicotyledonous plants. Gene **171:** 147–153

Pozueta-Romero J, Klein M, Houlné G, Schantz M-L, Meyer B, Schantz R (1995) Characterization of a family of genes encoding a fruit-specific wound-stimulated protein of bell pepper (*Capsicum annuum*): identification a new family of transposable elements. Plant Mol Biol **28:** 1011–1025

Pruvot G, Cuiné S, Peltier G, Rey P (1996) Characterization of a novel drought-induced 34 kDa protein located in the thylakoids of *Solanum tuberosum* L. plants. Planta **198:** 471–479

Rafia F (1995) Les plastes du fruit de Poivron: étude structurale et fonctionnelle des infrastructures mises en place au cours du mûrissement. Thèse d'Université, Biologie et Physiologie Cellulaire, Bordeaux 1, Bordeaux, France

Simpson DJ, Lee TH (1976) Plastoglobules of leaf chloroplasts of two cultivars of *Capsicum annuum*. Cytobios **15:** 139–147

Simpson DJ, Rahman FMM, Buckle KA, Lee TH (1974) Chemical regulation of plastid development II. Effect of CPTA on the ultrastructure and carotenoid composition of chromoplasts of *Capsicum annuum* cultivars. Aust J Plant Physiol **1:** 135–147

Sitte P, Falk H, Liedvogel B (1980) Chromoplasts. *In* FC Czgan, ed, Pigments in Plants. Gustav Fisher Verlag, New York, pp 117–148

Smirra I, Halevy AH, Vainstein A (1993) Isolation and characterization of a chromoplast-specific carotenoid-associated protein from *Cucumis sativus* corollas. Plant Physiol **102:** 491–496

Steinmüller D, Tevini M (1985) Composition and function of plastoglobuli. I. Isolation and purification from chloroplasts and chromoplasts. Planta **163:** 201–207

Verna A (1983) A simple quick-freezing device for ultrastructure preservation: evaluation by freeze-substitution. Biol Cell **49:** 95–98

Vishnevetsky M, Ovadis M, Itzhaki H, Levy M, Libal-Weksler Y, Adam Z, Vainstein A (1996) Molecular cloning of a carotenoid-associated protein from *Cucumis sativus* corollas: homologous genes involved in carotenoid sequestration in chromoplasts. Plant J **10:** 1111–1118

Young AJ, Wellings R, Britton G (1991) The fate of chloroplast pigments during senescence of primary leaves of *Hordeum vulgare* and *Avena sativum*. J Plant Physiol **137:** 701–705