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# Chromoplast Differentiation: Current Status and Perspectives

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**Chromoplasts are carotenoid-accumulating plastids conferring color to many flowers and fruits as well as to some tubers and roots. Chromoplast differentiation proceeds from preexisting plastids, most often chloroplasts. One of the most prominent changes is remodeling of the internal membrane system associated with the formation of carotenoid-accumulating structures. During the differentiation process the plastid genome is essentially stable and transcriptional activity is restricted. The buildup of the chromoplast for specific metabolic characteristics is essentially dependent upon the transcriptional activity of the nucleus. Important progress has been made in terms of mediation of the chloroplast-to-chromoplast transition with the discovery of the crucial role of the *Or* gene. In this article we review recent developments in the structural, biochemical and molecular aspects of chromoplast differentiation and also consider the reverse differentiation of chromoplasts into chloroplast-like structures during the regreening process occurring in some fruit. Future perspectives toward a full understanding of chromoplast differentiation include in-depth knowledge of the changes occurring in the plastidial proteome during chromoplastogenesis, elucidation of the role of hormones and the search for signals that govern the dialog between the nuclear and the chromoplastic genome.**

**Keywords:** Carotenoids synthesis • Chloroplast-to-chromoplast transition • Chromoplast metabolism • Chromoplast structure • Plastid genome • Regreening • Thylakoid breakdown

**Abbreviations:** ABA, abscisic acid; ARC5, accumulation and replication of chloroplasts5; ARF4, auxin response factor; CHRC, plastoglobulin carotenoid-associated protein; DDB1, UV-damaged DNA-binding protein 1; DET1, deetiolated 1; GFP, green fluorescent protein; MFP1, thylakoid-associated DNA-binding protein; ORFs, open reading frames; PVD1, plastid division1; PVD2, plastid division 2; ROS, reactive oxygen species.

## Introduction

During evolution, higher plants have adopted strategies to attract insects and mammals so as to facilitate flower pollination and seed dispersal. One of these strategies has been the development of bright colors, most often within a type of plastid called chromoplasts. Chromoplasts are responsible for the yellow, orange and red colors of many flowers and fruits. They are also present in some roots, such as carrots, and tubers such as sweet potatoes. Plastids are typical organelles unique to lower and higher plants that originate from the endosymbiotic integration of a photosynthetic prokaryote, cyanobacterium, into a eukaryotic ancestor of algae. The ancestors of plastids, chloroplasts, have diversified into a variety of other plastid types, including chromoplasts, to carry out specialized functions in nonphotosynthetic organs (Pyke 2007). Among nonphotosynthetic plastids, chromoplasts have received the most attention, as they accumulate pigments that are essential for the sensory quality of horticultural products. Since most of the pigments present in chromoplasts are carotenoids, the biochemistry and molecular biology of chromoplast differentiation has been largely devoted to the biochemistry and molecular biology of carotenoid formation (Camara et al. 1995, Bramley 2002). However, despite strong specialization, nonphotosynthetic plastids also carry out many other functions either specific to or remnants of chloroplastic functions in flowers (Tetlow et al. 2003) and fruits (Büker et al. 1998, Bouvier and Camara 2007). Biochemical and structural events during chromoplast differentiation have been reviewed, either specifically (Marano et al. 1993, Ljubesic et al. 1991) or within general papers on nongreen plastids (Thomson and Whatley 1980) and on plastid differentiation (Waters and Pyke 2004, Lopez-Juez 2007). Since then, novel information on the specific metabolic capacities of chromoplasts has been generated using high-throughput transcriptomic (Kahlau and Bock 2008) and proteomic approaches (Siddique et al. 2006, Barsan et al. 2010).

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This review focuses on recent data on the structural and molecular events occurring during the differentiation of chromoplasts to better understand how chromoplasts acquire their specific metabolic characteristics.

### Diversity of chromoplast structures

There is great variation in the morphology of chromoplasts, particularly in the structures that contain carotenoids. A classification into globular, membranous, tubular, reticuloglobular and crystalline has been proposed, although there is generally more than one type of pigment-containing body in a chromoplast (Ljubescic et al. 1991). Reticulotubular structures made of a reticulum of tubules are abundant in saffron, bananas and *Cucurbita maxima*, but vesicles and globules also coexist (Grilli-Caiola and Canini 2004). Some chromoplasts accumulate carotenoids as large crystals inside the lumina of thylakoid-like structures, such as in carrot roots and daffodil petals, and plastoglobuli containing small amounts of carotenoids are also present, although in small size and number (Kim et al. 2010). Mango fruit chromoplasts contain large and numerous globules as well as a network of tubular membranes. They can be considered as both the globular and reticulotubular types (Vasquez-Caicedo et al. 2006). Tomato fruit accumulates carotenoids, predominantly in the form of lycopene crystalloids in membrane-shaped structures (Harris and Spurr 1969). Chromoplasts of red pepper are characterized by a large number of globules with fibrillar extensions of carotenoid (Laborde and Spurr 1973). Different types of chromoplasts may coexist in the same organ. For instance, in *Thunbergia alata* flowers, mesophyll cells harbor chromoplasts of the tubulous type almost exclusively, while adaxial epidermal cells contain, in addition to the tubules, membrane and tubular reticulum structures (Ljubescic et al. 1996). A detailed supramolecular organization of the carotenoid-protein bodies of red pepper was described by Deruere et al. (1994), showing that the carotenoids, in association with tocopherols and quinines, are sequestered in the central core and are surrounded by a layer of polar lipids, which in turn are surrounded by an outer layer of the plastoglobulin fibrillin. In fact, fibrillin, which is highly expressed in ripening fruit, allows the sequestration of lycopene in the form of crystals within membrane structures. The sequestration prevents the otherwise detrimental effects of excess of carotenoids on cellular functions. Fibril initiation occurs in the plastoglobule. In chloroplasts where the level of carotenoids is low, the lipid:protein ratio is sufficient for the sequestration of carotenoids. The type of carotenoid-containing bodies therefore depends upon the lipid:protein ratio and the presence of proteins facilitating the assembly of carotenoids, such as fibrillin.

Chromoplasts that accumulate pigments during fruit ripening and flower development are functionally different from senescence-derived plastids. The yellow color of senescent plastids is due to the disappearance of chlorophyll and retention of carotenoids in the absence of de novo carotenoid biosynthesis. In addition, contrary to chromoplasts, they

undergo an extensive loss of plastidic DNA and are designed as gerontoplasts (Matile 2000).

### Changes in structure, morphology and composition of the plastid during chromoplast formation

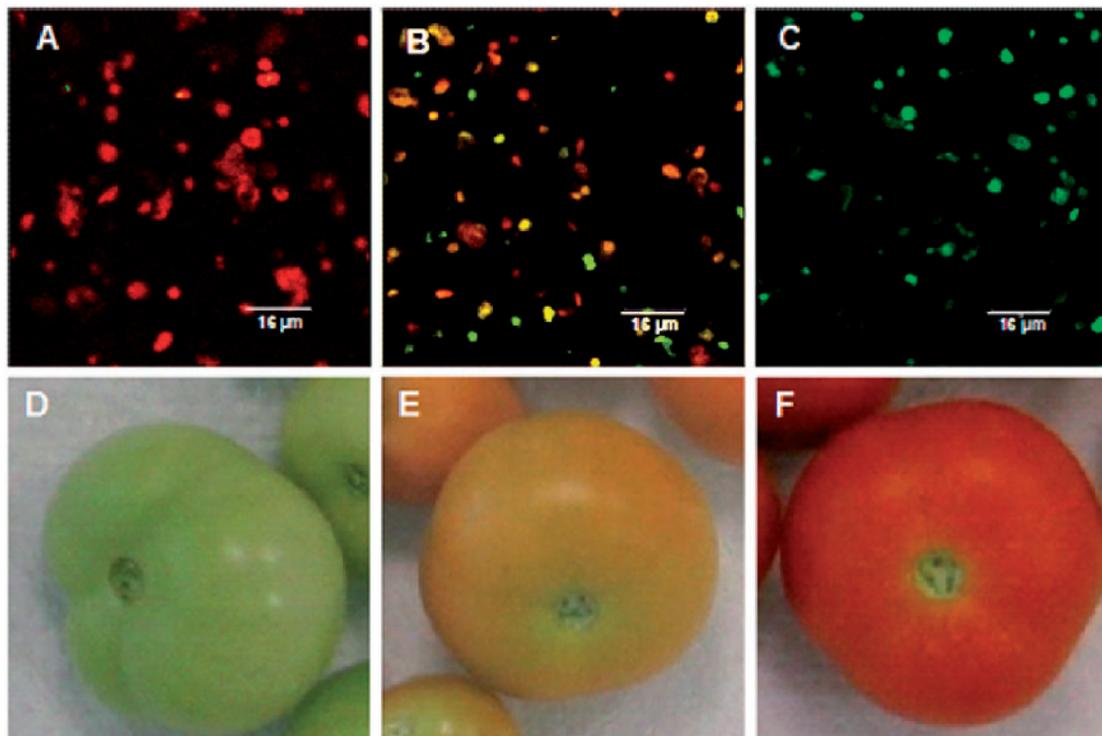
#### Changes in morphology and chlorophyll-carotenoids balance during chromoplast differentiation

Insights into the morphology of plastid differentiation and chlorophyll breakdown have been provided by confocal microscopy coupled with the plastid-located green fluorescent protein (GFP; Köhler and Hanson 2000, Waters et al. 2004, Forth and Pyke 2006). Pericarp cells in young green tomato fruit have a large number of regular-size plastids containing both chlorophyll and GFP, visualized by red autofluorescence and green fluorescence, respectively. As fruits ripen, the red fluorescence of plastids decreases in relation to chlorophyll degradation. Fully ripe pericarp cells possess a large number of chromoplasts, appearing as green due to the exclusive fluorescence of GFP in the absence of chlorophyll (Forth and Pyke 2006). The plastid size varies from the mature green to the fully ripe stages, chromoplasts being smaller than chloroplasts. At the breaker stage, plastids show considerable intracellular variability in size and differentiation status. The chloroplast–chromoplast transition events are presumably not simultaneous throughout the fruit or even within a cell, leading to a heterogeneous population of plastids. In addition, there are consistent differences in plastid size and appearance between inner mesocarp and outer mesocarp cells of tomato fruit. Chromoplasts of the outer mesocarp have an oblong, needle-like appearance, whereas chromoplasts in the inner mesocarp are much larger and have an ovoid shape (Waters et al. 2004).

The transition from chloroplast to chromoplast can be visualized during the ripening process by exploiting the autofluorescence of chlorophyll and carotenoids of purified plastid fractions (Fig. 1). At the mature green stage, all plastids are chloroplasts and the emitted fluorescence gives a red color due to the dominance of chlorophyll (Fig. 1A). At the breaker stage, the population of plastids is highly heterogeneous, but by using adapted isolation procedures, intermediate chloro-chromoplasts can be obtained containing both chlorophyll and carotenoids so that the emitted fluorescence gives a yellowish color due to the merging of red (chlorophyll) and green (carotenoid) fluorescence (Fig. 1B). At the fully ripe stage, only fully developed chromoplasts are present, and appear green, corresponding to the autofluorescence of carotenoids (Fig. 1C).

#### Preexisting plastids from which chromoplasts originate

Different forms of plastids can be generated by interconversions of preexisting plastid types. A cycle of plastid development



**Fig. 1** Confocal images of chloroplast (A), chloroplast initiating transition (B) and mature chromoplast (C) suspensions isolated from mature green (D), breaker (E) and fully ripe (F) tomatoes. Images are overlays of chlorophyll autofluorescence and carotenoid autofluorescence emitted at wavelengths between 740 and 750 nm (red) and between 500 and 510 nm (green), respectively, when they are excited using the 488 nm line from the argon laser. Structures containing mainly chlorophyll appear red, those containing only carotenoid appear green, and those containing both chlorophyll and carotenoid appear orangey red/yellow. Scale bars = 16  $\mu$ m.

interrelationships has been suggested (Whatley 1978). Chloroplast differentiation from proplastids, under the control of light, is one of the best-known interconversions (Lopez-Juez and Pyke 2005). Chromoplasts may arise directly from proplastids (e.g. in carrot roots; Ben-Shaul and Klein 1965), indirectly from chloroplasts (e.g. in ripening fruit; Bathgate et al. 1985) or from amyloplasts (e.g. in saffron flowers; Grilli-Caiola and Canini 2004; or tobacco floral nectaries; Horner et al. 2007). An interesting example of plasticity exists in *Arum italicum* berry fruit, where the various steps of maturation and ripening are associated with a sequence of transitions involving amyloplast, chloroplast and chromoplast (Bonora et al. 2000).

Analysis of plastid division in tomato fruit revealed that the majority of plastid division by binary fission occurs during the fruit enlargement stages when the plastids are present as chloroplasts. The plastid number remains fairly constant once ripening begins (Cookson et al. 2003). Replication of chromoplasts is occasionally observed, such as in pepper fruit (Leech and Pyke 1988) and *Forsythia suspensa* petals (Sitte 1987). Replication by budding and fragmentation has also been observed in the *suffulta* mutants, in which a heterogeneous population of plastids exist (Forth and Pyke 2006). In agreement with the absence or low rate of division in regular tomato chromoplasts, only a few members of the plastid division

machinery have been encountered in the proteome (Barsan et al. 2010). Several homologs of the 3 FtsZ proteins of *Arabidopsis* have been detected, but the other parts of the plastid division machinery (Pyke 2007), such as ACCUMULATION AND REPLICATION OF CHLOROPLASTS5 (ARC5), PLASTID DIVISION1 (PVD1) and PLASTID DIVISION2 (PVD2), were absent.

### Internal membrane remodeling during chromoplast formation

Electron microscopy studies carried out in red pepper by Spurr and Harris (1968) have shown that remodeling of the internal membrane system starts with lysis of the grana and the inter-granal thylakoids. Some small and loosely aggregated groups of initial thylakoids still persist at advanced stages of ripening. In parallel, new membrane systems are formed consisting in organized membrane complexes called thylakoid plexus by Spurr and Harris (1968) and thylakoid sheets. These early observations are consistent with most recent data (Simkin et al. 2007) showing that during the chloroplast–chromoplast conversion in tomato fruit, the thylakoid disassembly is associated with the synthesis of new membranes that are the site for the formation of carotenoid crystals. These newly synthesized membranes do not derive from the thylakoids, but rather from

vesicles generated from the inner membrane of the plastid (Simkin et al. 2007).

The loss of thylakoid integrity revealed by ultrastructure studies corresponds to a late event of chromoplast development that is visible well after the loss of thylakoid-associated metabolic functions. For instance, in the flower bud of *Lilium longiflorum*, the rapid decline of photosynthetic activity during the chloroplast–chromoplast transition occurs well before any observable loss of thylakoid integrity and reduction of chlorophyll (Clément et al. 1997). The metabolic machineries are not affected at the same rate. Within the photosynthetic apparatus, photosystem II integrity was preserved longer than the rest of the machinery (Juneau et al. 2002). Interestingly, the loss of thylakoid integrity during tomato fruit ripening is associated with a large decrease of a thylakoid-associated DNA-binding protein, MFP1, which is supposed to participate in the development of the thylakoid membrane (Jeong et al. 2003).

### Role of plastoglobules and plastoglobulins in the storage of carotenoids

During the chloroplast–chromoplast transition, an increase in the size and number of plastoglobuli is generally observed (Harris and Spurr 1969). Microscopy studies demonstrated that plastoglobules arise from a blistering of the stroma-side leaflet of the thylakoid membrane, predominantly along highly curved margins (Austin et al. 2006).

There is experimental evidence that plastoglobulins participate in the sequestration of carotenoids and in the biogenesis of chromoplasts (reviewed by Bréhélin and Kessler 2008). The observation that suppression in tomato plants of the plastoglobulin carotenoid-associated protein (CHRC) results in 30% reduction of carotenoids in tomato flowers provided the first evidence for the role plastoglobulin chromoplast differentiation (Leitner-Dagan et al. 2006). Other evidence included the overexpression in tomato of a pepper plastoglobulin, fibrillin, that caused an increase in carotenoid and carotenoid-derived flavor volatiles (Simkin et al. 2007). In addition, the loss of thylakoids was delayed during the chloroplast–chromoplast transition and the plastids showed a typical chromoplastic zone contiguous with a preserved chloroplastic zone. It is concluded that fibrillin plays a role in thylakoid disorganization during chromoplast formation.

Plastoglobules not only act as lipid storage bodies, but they also participate in some metabolic pathways (Bréhélin and Kessler 2008). Analysis of the proteome of red pepper plastoglobules indicated the presence of several proteins involved in the synthesis of carotenoids, including  $\zeta$ -carotene desaturase, lycopene  $\beta$ -cyclase and two  $\beta$ -carotene  $\beta$ -hydroxylases (Ytterberg et al. 2006). Also,  $\zeta$ -carotene desaturase has been detected in the proteome of tomato chromoplasts (Barsan et al. 2010).

The chloroplast–chromoplast transition is shown in Fig. 2, in which remodeling of the internal membrane system and formation of carotenoid storage structures are represented.

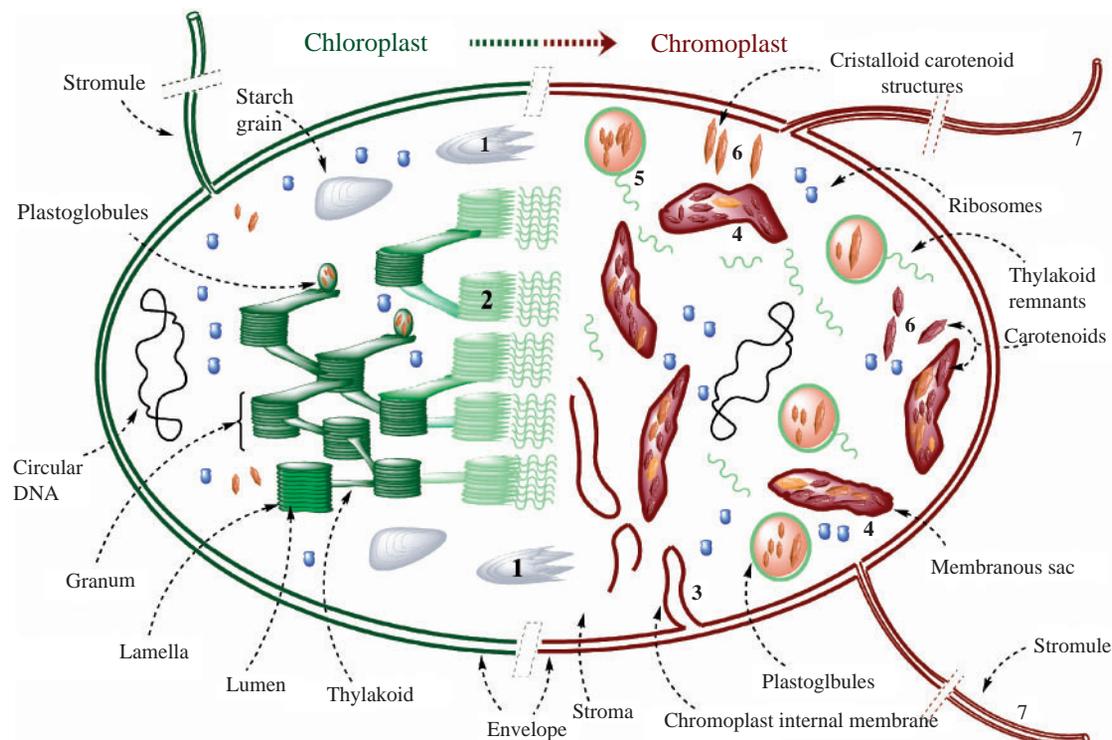
### Changes in stromule morphology during chromoplastogenesis

Stromules are motile protrusions emanating from the plastid membrane into the cytoplasm. Microscopy techniques coupled with GFP have revealed that the importance of stromules generally increases with the progress of fruit ripening in tomato (Waters et al. 2004). However, there are some differences between tomato tissues. Long stromules are associated with plastids that are further apart, whereas short stromules are present in cells with a high density of plastids. In the outer mesocarp, where cells have a high density of plastids, stromules are short and form a complex chromoplast network (Pyke and Howells 2002). In the inner mesocarp, the density of plastids is lower and stromules are longer and their number and length increases during ripening (Waters et al. 2004). Once the fruit begins to ripen, stromules increase in number and length, at least in the inner mesocarp, probably to provide a greater import area for novel proteins (Kwok and Hanson 2004), particularly those involved in carotenoid biosynthesis and chromoplast differentiation. Sometimes, free broken stromules detached from the plastid appear throughout the cytoplasm of green fruit as small vesicles containing only GFP. They may have the potential to develop into full chromoplasts (Waters et al. 2004). The *green flesh* mutation, in which plastid differentiation is incomplete, and the *rin* mutation, in which the ripening process is blocked, result in a reduction of stromule formation (Waters et al. 2004).

### Characteristics and stability of the plastid genome during differentiation into chromoplasts

The plastid genome (plastome) of the tomato fruit has the same basic characteristics as the majority of the plastomes that have been sequenced. The size of the tomato fruit plastome is 155,461 bp and comprises a large and a small single-copy region intercalated by two inverted repeats, IR<sub>a</sub> and IR<sub>b</sub> (Kahlau et al. 2006). Annotation indicated the presence of 114 genes and conserved open reading frames (ORFs) divided into three major categories (Sugiura 1992): (i) photosystem-related genes; (ii) genetic system genes, including genes encoding ribosomal proteins, tRNA and a plastid RNA polymerase; and (iii) the hypothetical chloroplast reading frames (*ycfs*), a group of conserved sequences, some of them of unknown function, but essential for plastids activity (Ravi et al. 2008).

Comparison by restriction enzyme analysis of the DNA of chloroplasts from leaves and chromoplasts from tomato fruit revealed the absence of rearrangements, losses or gains (Hunt et al. 1986). Subtle changes in DNA, such as increased methylation of cytosine, have been suggested upon analysis by liquid chromatography of tomato chromoplasts (Kobayashi et al. 1990). However, this observation was not confirmed by Marano and Carrillo (1991), who found that the patterns of DNA methylation assessed after restriction and hybridization analysis with



**Fig. 2** Schematic representation of the chloroplast–chromoplast transition. The scheme shows the breakdown of starch granules (1) and of grana and thylakoids (2); the synthesis of new membrane structures from the inner membrane envelope of the plastid (3) leading to the formation of carotenoid-rich membranous sacs (4); the increase in the number and size of plastoglobules (5); the appearance of carotenoid-containing crystalloids (6); and the increase in the number of protrusions emanating from the plastid envelope, called stomules (7).

DNA probes did not differ significantly between chloroplasts of mature green tomatoes and chromoplasts of red ripe fruit. Since structural and methylation changes in DNA have not been firmly established, their role in plastid switching remains uncertain.

### Importance of transcriptional and translational activity during chromoplast differentiation

The expression pattern of few plastid localized genes has been studied. As expected, genes involved in carotenoid biosynthesis, such as *lycopene  $\beta$ -cyclase* (*CYCB*), are up-regulated during chromoplast formation in many plants, including citrus fruit (Alquezar et al. 2009); the wild species of tomato, *Solanum habrochaites* (Dalal et al. 2010); saffron (Ahrazem et al. 2010); papaya fruit (Blas et al. 2010); and carrot (Chen et al. 2001). On the contrary, genes involved in photosynthetic activity are generally down-regulated during chromoplast formation (Cheung et al. 1993). Surprisingly, up-regulation of the large subunit of *ribulose-1,5-bisphosphate carboxylase/oxygenase* and the 32 kD *photosystem II quinone binding protein* genes has been observed in chromoplasts of squash fruits (*Cucurbitae pepo*; Obukosia et al. 2003), indicating that the expression pattern of these photosystem genes may be

regulated independently from the plastid differentiation processes.

A comprehensive study of chromoplastic transcriptome was carried out by Kahlau and Bock (2008) showing that the global transcriptional activity remains almost the same during chromoplast differentiation, except for a limited number of genes, including (i) *accD*, which encodes a subunit of the acetyl-CoA carboxylase involved in fatty acid biosynthesis; (ii) *trnA* (tRNA-ALA); and (iii) *rpoC2* (RNA polymerase subunit). During fruit ripening a reduction of translational activity has been observed by comparison of polysome-associated plastidial mRNA levels between fruit chloroplasts and chromoplasts. Rather than a decrease in transcription, plastid translation appears to be the main factor that contributes to down-regulation of chromoplast proteins during fruit plastid differentiation. Another line of evidence to support this hypothesis is that the activity of both the nuclear-encoded and plastid-encoded RNA polymerases undergoes little change during the transition. Likewise, RNA splicing activity of the plastid, a possible mechanism contributing to the regulation of gene expression, exhibits insignificant changes during tomato fruit ripening. In any case, transcriptional and translational activities of the plastid make a limited contribution to chromoplast differentiation. The large majority of the proteins present in the plastid are encoded

by nuclear genes so that transcriptional activity in the nucleus and translocation of proteins into the plastid are of primary importance for the buildup of chromoplast metabolism. Proteins related to the biosynthesis of fatty acids, amino acids, carotenoids, vitamins, hormones, aroma volatiles and other substances have been encountered in the chromoplastic proteome of tomato (Barsan et al. 2010). These proteins participate in giving the fruit important sensorial characteristics such as color and aroma. Many of the corresponding genes are regulated by the plant hormone ethylene and therefore participate in the transcriptional regulation of the fruit ripening process in general (Giovannoni 2001, Pirrello et al. 2009). As shown in the following paragraph, some of the nuclear-localized genes play a crucial role in chromoplast differentiation.

### Genes involved in chromoplast differentiation and development of carotenoid storage structures

Due to increased expression during the chloroplast–chromoplast transition, some genes have been suspected to play a role in chromoplastogenesis. Such is the case for the *early light-inducible protein (ELIP)* gene, which has homology with light-harvesting complex proteins and whose expression is high during the breaker/turning ripening stages in tomato. However, no direct evidence for a role of *ELIP* or other genes in chromoplast differentiation had been provided until the discovery of the cauliflower *Or* gene (Lu et al. 2006). The dominant mutation *Or* confers an orange pigmentation with accumulation of  $\beta$ -carotene mostly in the inflorescence of cauliflower without significantly affecting the expression of carotenoid biosynthetic genes (Li et al. 2001). Chromoplasts differentiate in the *OR* mutant and develop membranous inclusions of carotenoids resembling those of carrot roots. In addition, there was an arrest in plastid division, and for this reason only one or two chromoplast are present in the affected cell (Paolillo et al. 2004). Chromoplast differentiation occurs mostly in the inflorescence tissues, but not in the leaves, suggesting that tissue-specific expression is regulated at the transcriptional or posttranscriptional levels. The *Or* transgene introduced in a tuber-specific manner into potato induces a sharp increase in the accumulation of carotenoids, again without affecting the expression of endogenous carotenoid biosynthetic genes (Lu et al. 2006, Lopez et al. 2008). The *Or* gene is nuclear-localized and encodes a DnaJ-like cochaperone containing a cysteine-rich domain lacking the J-domain (Lu et al. 2006). The role of the DnaJ proteins is to interact with Hsp70 chaperones to perform protein folding, assembly, disassembly and translocation. The absence of phenotype upon RNAi silencing suggests that *Or* is not a loss-of-function mutation, and putative interaction with Hsp70 chaperones indicates that it might be a dominant-negative mutation (Giuliano and Diretto 2007). Altogether, these data show

that the *Or* gene is not directly involved in carotenoid biosynthesis, but rather causes a metabolic sink for carotenoid accumulation by inducing the formation of chromoplasts (Li and van Eck 2007).

The role of some genes in the formation of carotenoid storage structures has been explored. Overexpression of phytoene synthase gene causes carotenoid crystal formation on nongreen tissues of *Arabidopsis*, but not in green tissues, indicating a fundamental difference in carotenoid storage mechanisms (Maass et al. 2009). Therefore the sequestration of carotenoids into crystals resulting from high activity of phytoene synthase can happen in the absence of a chromoplast developmental program, such as in *Arabidopsis*, as a consequence of enhanced carbon flux through the pathway. High phytoene synthase has also been associated with  $\beta$ -carotene accumulation in orange carrot roots (Maass et al. 2009).

### Metabolic activities of chromoplasts

The metabolic activity of chromoplasts has already been reviewed (Neuhaus and Emes 2000, Bouvier and Camara 2007). Here we provide an overview of the main features and include some recent data. When chromoplasts derive from chloroplasts, the most obvious biochemical change is the loss of chlorophyll and photosynthetic activity associated with the down-regulation of photosynthetic gene expression (Piechulla et al. 1985). Another major feature of chromoplast metabolism is the accumulation of pigments. Several reviews have been dedicated to the biosynthesis of carotenoids in fruits and flowers (Bramley 2002, Fraser and Bramley 2004, Lu and Li 2008). However, they are also the site for the synthesis of sugars, starches, lipids, aromatic compounds, vitamins (riboflavin, folate, tocopherols) and hormones (Neuhaus and Emes 2000, Barsan et al. 2010). For sustaining biosynthetic activities, sugars are imported from the cytosol by a plastid-localized glucose transporter (Bouvier and Camara 2007), but the use of endogenous sugars resulting from starch degradation cannot be excluded. Proteins of starch biosynthesis and degradation remain present in tomato chromoplasts (Barsan et al. 2010). Calvin cycle enzymes have been measured in plastids isolated from sweet pepper, and their activities were generally greater in chromoplasts than in chloroplasts (Thom et al. 1998). In tomato, the activity of Calvin cycle enzymes has also been observed (Obiadalla-Ali et al. 2004). In association with the persistence of the active oxidative phosphate pathway (Tetlow et al. 2003, Bouvier and Camara 2007, Barsan et al. 2010), adenosine triphosphate (ATP) and reducing power are produced that also participate in sustaining the metabolic activities of chromoplasts. Another interesting feature of chromoplasts is the presence of highly active antioxidant system. The level of glutathione and ascorbate in the plastids isolated from pepper fruit increase during fruit ripening in parallel with the activity of the enzymes of the ascorbate glutathione cycle and superoxide dismutase (Marti et al. 2009). High activity of the antioxidant system in the chromoplasts could play a role in protecting

plastid components such as carotenoids against oxidation, but also in mediating signaling between chromoplasts and the nucleus. Reactive oxygen species (ROS) are thought to participate in plastid to nucleus communication (Kleine et al. 2009, Galvez-Valdivieso and Mullineaux 2010). Plastid-generated ROS are known to up-regulate the transcription of genes of carotenoid biosynthesis (Bouvier et al. 1998).

### Reversible differentiation of chromoplasts

Reversible differentiation of plastids is another aspect of plasticity of the organelle. Preberg et al. (2008) quote a number of situations where regreening of tissues occurs as a consequence of redifferentiation of gerontoplasts, etioplasts or chromoplasts into chloroplasts. The phenomenon is truly a redifferentiation process without any evidence of de novo generation of plastids or plastid division. In the case of chromoplasts, the best known example of reversal to chloroplasts is that of citrus fruits (Thomson et al. 1967), but the phenomenon also exists in other species such in cucumber fruit (Preberg et al. 2008). Ultrastructural aspects of the reversion of chromoplasts to chloroplasts have been described in the subepidermal layer of fruit of *Cucurbita pepo* (Devide and Ljubecic 1974). During regreening, the globular-type chromoplasts with numerous plastoglobules and small vesicle-like fragments of thylakoids undergo a disappearance of plastoglobules and the formation of new thylakoids. Thylakoids arise from both preexisting vesicles and from the invagination of the inner membrane of the plastid to form grana structures, leading to normal chloroplast structure and photosynthetic activity. Similar reconstitution of the thylakoid system has been described recently along with more details during redifferentiation of chloroplasts in cucumber fruit (Preberg et al. 2008). In this case, the plastoglobules persisted during the entire process and remnants of the degraded thylakoid system formed large membrane-bound bodies that later participated in the reformation of thylakoids.

Light is probably the most important factor of regreening via phytochromes, however, nutritional factors are also involved. Warm temperatures, nitrogen fertilization and gibberellins stimulate regreening of citrus peel, while an abundance of sucrose tends to inhibit this process (Huff 1983). Very little information is available on the molecular mechanisms of reversal from chromoplasts to chloroplasts. However, there is evidence that gibberellic acid, which stimulates the greening process, reduces the expression of carotenoid biosynthetic genes, phytoene synthase, phytoene desaturase and  $\beta$ -carotene hydroxylase in orange flavedo (Rodrigo and Zacarias 2007). In clementines, gibberellins and nitrate that favor regreening reduced the expression of not only phytoene synthase, but also of the chlorophyll-degrading gene pheophorbide *a* oxygenase (Alos et al. 2006), indicating that regreening involves both repression of carotenoid biosynthesis and reduction of chlorophyll breakdown. No information is available yet on the expression of genes involved in the biosynthesis of photosystems and chlorophyll.

### Mutants with altered chromoplast development

We have already mentioned the *Or* mutant of cauliflower, which has allowed the isolation of a gene controlling the differentiation of chromoplasts. Here we briefly examine other mutants, mostly of tomato, showing, among other phenotypes, altered plastid development. Despite the pleiotropic effects of the mutation, these mutants represent useful tools for the identification of the molecular players involved in chromoplast biogenesis.

Compared to wild type, the natural mutants *HIGH PIGMENT 1* and *2* (*hp1* and *hp2*) have dark-green immature fruits and accumulate higher levels of carotenoids in ripe fruits (Yen et al. 1997, Mustilli et al. 1999). The *hp1* mutant codes a homolog of the *Arabidopsis* UV-DAMAGED DNA-BINDING PROTEIN 1 (DDB1) protein, which is predicted to interact with the nuclear factor DEETIOLATED 1 (DET1) (Liu et al. 2004), while the *hp2* codes a tomato ortholog of *Det1* (Mustilli et al. 1999). Ripe fruits of both mutants contain more and bigger chromoplasts per cell than wild-type fruits. The product of the *Det1* gene is part of the CUL4-based E3 ubiquitin ligase complex of the proteasome (Bernhardt et al. 2006, Wang et al. 2008). In the *HIGH PIGMENT 3* mutant (*hp3*), which has the same phenotype as *hp1* and *hp2*, the level of abscisic acid (ABA) is lower than in wild-type plants, suggesting that ABA deficiency could be an important factor for the development of the phenotype. This hypothesis is further sustained by the analysis of two other ABA-deficient tomato mutants, *flacca* and *sitiens*, that harbor similar alterations in plastid development (Galpaz et al. 2008).

The mutation in the locus *suffulta* provokes changes in the division of plastids in tomato plants, generating cells with giant chloroplasts but with low chlorophyll content. An unusual process of plastid division occurs during the chloroplast–chromoplast transition, characterized by budding and plastid fragmentation into small vesicles. This results in a heterogeneous population of chromoplasts at different development stages, with some of them keeping the chloroplast structure (Forth and Pyke 2006). The molecular identity of the gene responsible for the *suffulta* phenotype is unknown. It could be a component of the plastidial division machinery or could regulate the division process. It could also participate in the differentiation of chromoplasts.

In addition to light, phytohormones have been reported to play an important role in controlling chloroplast/chromoplast formation and stability during tomato fruit development. It is well known that tomato mutants and transgenic lines, impaired in elements of the ethylene signaling transduction cascade such as the ethylene receptor NR (Wilkinson et al. 1995), present altered pigmentation. Down-regulation of ARF4, an auxin response factor formerly named DR12, resulted in a dark-green phenotype and blotchy ripening of tomato fruit (Jones et al. 2002). In the ARF4 down-regulated lines, the outer pericarp tissue displayed a higher number of chloroplasts per cell and a dramatic increase in grana formation. Interestingly, in contrast

to *hp* mutants, the dark-green phenotype in ARF4-inhibited lines is confined to the fruit. The treatment of tomato fruits with fluridone, an inhibitor of ABA synthesis, has inhibitory effects on carotenoid accumulation (Zhang et al. 2009). It was also reported that exogenous treatment with cytokinin can mimic the *hp* mutant phenotype (Mustilli et al. 1999) and that *cytokinin hypersensitive Arabidopsis* mutants show increased chloroplast development (Kubo and Kakimoto 2000). The blotchy ripening phenotype was also induced by ectopic expression in tomato lines of the *ipt* gene from the Ti plasmid of *Agrobacterium tumefaciens* (Martineau et al. 1994). In this latter case, fruit displayed higher levels of cytokinin, and during ripening the fruit exhibited an altered phenotype, with green patches remaining within a deep red background.

The *rin* tomato mutant harbors a nonfunctional MADS-box transcription factor that is essential for fruit ripening (Vrebalov et al. 2002). The number of chromoplasts per cell in *rin* fruits at the breaker stage is much higher than the wild type, and the plastids are very small with few stromules. Whether the *RIN* gene is a direct regulator of the plastid transition in tomato fruit or the lack of ethylene synthesis in *rin* fruits is responsible for the abnormal chromoplast biogenesis remains unclear.

*Green flesh* and *chlorophyll retainer* are mutants of tomato and pepper, respectively, that have no ability to degrade chlorophyll during fruit ripening, but are able to synthesize carotenoids, resulting in brown color fruits. The plastids in the ripe fruit of these two mutants have remnants of thylacoidal membranes and formation of plastoglobuli, suggesting that the conversion of chloroplasts to chromoplasts is not completely concluded. The level of the carotenoids in the mutants is lower than in wild-type fruits, and several photosystem genes, like *rbcl* and *cab*, are up-regulated. Barry et al. (2008) indicated the possibility that this mutation is due to an impaired gene product linked to the chlorophyll degradation pathway.

All mutations described above show evidence that chromoplast formation is a complex event that involves not only factors expressed during ripening, but also developmental factors and hormones like auxin, cytokinin, ABA and ethylene. Two processes seem to be important for normal chromoplast biogenesis, chloroplast division and the biosynthesis of carotenoids. However, the way in which these processes are coordinated by nuclear and plastid gene expression remains unclear and represents a challenge for future studies.

### Future perspectives

The functional genomics tools will allow new insights into the mechanisms of chromoplast development. As revealed by a comprehensive survey with the new mass spectrometry technologies, the number of proteins assigned to the chromoplast proteome (Barsan et al. 2010) is comparable to that of the chloroplast proteome (Ferro et al. 2010). The development of specific protocols for isolating plastids at different stages of

differentiation associated with the use of comparative proteomic methods represent an interesting perspective toward the uncovering of target proteins that play a role in the chromoplast differentiation process.

Moreover, the combination of transcriptomic and proteomic data will allow identification of molecular events that are regulated at the transcriptional, posttranscriptional or posttranslational levels. In particular, the identification of phosphorylation and other types of protein modifications by proteomic analysis will provide useful information on the levels of regulation of plastid metabolic activity during chloroplast–chromoplast transition steps.

While a number of experimental data support the role of phytohormones in regulating plastid differentiation and evolution during ripening, the underlying mechanisms remain unclear. Also, although many hormones such as ethylene, auxin, cytokinin and ABA seem to take part in the regulation of the chloroplast–chromoplast transition, the extent of the cross talk between hormones to tune the process is unknown.

It is admitted that the expression of many genes targeted to plastids is regulated through a dialog between the nucleus and the plastid. These signals, whether environmental (temperature, light, etc.) or developmental, are supposed to comprise ROS, carotenoids, carbohydrates and hormones (ABA, jasmonates). The presence of a plastid–nucleus dialog is verified by the fact that exposure of the chloroplast to tagetitoxin, a specific inhibitor of plastidial RNA polymerase (Rapp and Mullet 1991), or lincomycin, a specific inhibitor of plastid peptidyl transferase (Mulo et al. 2003), decreases the accumulation of plastid-targeted nuclear transcripts. However, the contribution of these signals to the expression of specific genes is far from being fully understood (Kleine et al. 2009). In addition, most of the studies dealing with nucleus-to-plastid signaling have been carried out with chloroplasts. Whether some of these mechanisms are active in nonphotosynthetic plastids, such as chromoplasts, remains an open question. We are still far from a clear understanding of the dialog between the nuclear and the plastidial genome in mediating the differentiation of chromoplasts, and thus we are far from controlling developmental processes such as fruit ripening and flower development.

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