



Research article

Characterization under *quasi*-native conditions of the capsanthin/capsorubin synthase from *Capsicum annuum* L

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ABSTRACT

Chromoplasts are typical plastids of fruits and flowers, deriving from chloroplasts through complex processes of re-organization and recycling. Since this transition leads to the production of reactive species, chromoplasts are characteristic sites for biosynthesis and accumulation of carotenoids and other antioxidants. Here, we have analysed the chromoplast membranes from *Capsicum annuum* L. fruits, finding a significant expression of the capsanthin/capsorubin synthase. This enzyme was isolated by a very mild procedure allowing its analyses under *quasi*-native conditions. The isolated complex appeared as a red coloured homo-trimer, suggesting the retention of at least one of the typical carotenoids from *C. annuum*. Moreover, the protein complex was co-purified with a non-proteinaceous fraction of carotenoid aggregates carrying a high molecular weight and separable only by Size Exclusion Chromatography. This last finding suggested a relationship between the carotenoids synthesis on chromoplast membranes, the presence, and storage of organised carotenoids aggregates typical for chromoplasts. Further MS analyses also provided important hints on the interactome network associated to the capsanthin/capsorubin synthase, confirming its functional relevance during ripening. Results are discussed in the frame of the primary role played by carotenoids in quenching the growing oxidative stress during fruits ripening.

1. Introduction

Fruit ripening is an ensemble of complex physiological processes guided by hormonal and environmental factors (White, 2002; Ozga and Reinecke, 2003; Prasanna et al., 2007). During development, fruits' cells undergo extreme morphologic and metabolic changes, among which there is the chloroplast-to-chromoplast transition (Summer and Cline, 1999; Li and Hui, 2013; Sun et al., 2018). This process sees the dismantling of thylakoid membranes and the recycling of their components, which are mainly exported and used in other compartments of the plant. At the same time important processes associated to the biosynthesis of antioxidants, antibiotic molecules, and flavouring components also take place (Spurr and Harris 1968; Howard et al., 2000; Sun et al., 2018). During ripening, the fruit passes from an auto- to a heterotrophic metabolism, becoming a “passive” organ subject to respiration of organic acids and accumulation of sugars (Hall, 1977; Seymour et al., 1993; Fisher et al., 2012; Batista-Silva et al., 2018). This exposes

ripening fruits to oxidation events, with concomitantly increased levels of Reactive Species (RS). Therefore, during ripening, RS quenching assumes a pivotal role and must be finely controlled in order to reach a precise species-specific synchrony between fruits and seeds maturity (Bouvier et al., 1998; Prasanna et al., 2007; Osorio et al., 2013; Airaki et al., 2015; Kumar et al., 2016; Corpas et al., 2018). In this context, the complex mechanism of RS scavenging, finely regulated by hormones, makes use of carotenoids, flavonoids, and derivatives of phenylpropanoids (White, 2002; Blokhina et al., 2003; Prasanna et al., 2007; Wang et al., 2013). Among these groups of molecules, carotenoids have the strongest scavenging efficiency and they are the main class of antioxidants in ripened fruits. This is particularly true in the case of the *Capsicum annuum* fruits, for which the development and physiology has been extensively studied (Kilcrease et al., 2013; Chaki et al., 2015; Palma et al., 2015), especially in light of their nutritional, medical, and economic importance worldwide (Palevitch and Craker, 1996; Palma et al., 2015; Arimboor et al., 2015; Fernández-Bedmar and Alonso-

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Moraga, 2016). In red pepper fruits, the relationship between ripening and carotenoid levels is straightforward. In fact, the conversion from chloroplast to chromoplast is marked by an increase in the rate of the carotenoid synthesis bringing about their massive accumulation (Spurr and Harris 1968; Hornero-Mendez et al., 2000). This event is coupled with a complete remodelling of the thylakoid membranes and the building of proteinaceous substructures aimed at carotenoid sequestration and accumulation (Sun et al., 2018). It is possible to distinguish between five types of chromoplasts: membranous, globular, tubular, fibrillar, and crystalline, based on the strategy followed during carotenoid sequestration, in fruits and flowers. *C. annuum* fruits are well known for the high amounts of fibrillar chromoplasts in their cells (Simpson and Lee, 1976; Deruère et al. 1994; Kilcrease et al., 2013) and for the accumulation of carotenoids in these organelles, through an organised packing, mediated and directed by the lipoprotein fibrillin (Deruère et al. 1994). In these fruits, the transitions of colours from green to dark-red is driven by the accumulation of several carotenoids, among which the capsanthin and the capsorubin, products of the enzyme capsanthin/capsorubin synthase (CCS), are the main constituents (Valadon and Mummery 1976; Schweiggert et al. 2005; Siddique et al., 2006; Ha et al., 2007; Mialoundama et al., 2010). Capsorubin and capsanthin originate from the CCS through a long pathway starting from the common carotenoid's precursor phytoene (Gómez-García and Ochoa-Alejo, 2013). By several steps of desaturation, isomerization, and cyclization lycopene and β -carotene are produced. Eventually, by this long pathway β -carotene is converted into the xanthophyll zeaxanthin, the direct precursor of the CCS substrates antheraxanthin and violaxanthin (Gómez-García and Ochoa-Alejo, 2013; Yuan et al., 2015). The CCS was firstly isolated in 1994 by Bouvier and coworkers, who found the enzyme as a monomer on the chromoplast membrane fraction isolated from *C. annuum* fruits (Bouvier et al. 1994). Since that time, the growing physiological importance of this enzyme, its gene, and its products emerged through the characterization of its properties and peculiarities (Mialoundama et al., 2010; Li and Hui, 2013). With the aim to maximize the native CCS integrity, we have isolated the chromoplast membranes under very mild conditions and exposed them to mild detergents for short times. The subsequent CCS isolation was performed by a direct chromatographic procedure on the solubilized membranes; finally, the enriched CCS samples were separated from a non-proteinaceous co-solubilized fraction in a second chromatographic step. This procedure clearly allowed the isolation of the CCS, finding it exclusively in its homo-trimeric form, thus stabilizing and preserving supramolecular interactions. Furthermore, we have not only observed that the CCS occurs in increasing amounts during ripening in *C. annuum* L., but also that it is the most highly expressed protein complex in the whole chromoplast membranes proteome. Finally, MS analyses also showed the co-presence of other proteins that were found only in small amounts, suggesting them as possible components of the CCS interactome network. These findings shed new light on our understanding of the carotenoids' dynamics and trafficking during fruit ripening. We discuss these results in the context of the role of capsorubin and capsanthin in quenching the growing oxidative stress that occurs during fruit ripening in *C. annuum*.

2. Materials and methods

2.1. Plant material

Capsicum annuum L. (Red Bell Pepper) were grown in green houses at the constant temperature of 25 °C under natural illumination and at 60% of relative air humidity.

2.2. Isolation and solubilisation of chromoplast membranes

Chromoplast membranes were isolated at 4 °C in the dark by modifying a procedure for thylakoid membranes isolation from leaves

(Haniewicz et al., 2013). Briefly, 100 g of fruits were washed in distilled water and dried in paper towels. Seeds and placenta tissues were carefully removed. Weighed fruits were mixed in a ratio 1:1 (g/mL) with Grinding Buffer (GB - 50 mM MES pH 6.5; 10 mM $MgCl_2 \cdot 6H_2O$; 10 mM $CaCl_2 \cdot 2H_2O$) and blended for 15". The resulting suspension was filtered with a filter composed of two muslin layers with an interposed layer of cotton. The suspension was subsequently centrifuged at 5000 g for 10' at 4 °C and the obtained pellet was resuspended in a small volume of Resuspension Buffer (50 mM Na phosphate pH 7.8). The obtained suspension was homogenized on ice and membranes were solubilized for 20' under slow stirring at 4 °C in 20 mM n-dodecyl- β -D-maltoside (β -DDM). After a subsequent centrifugation step (at 30,000 g for 10' at 4 °C), the pellet was discharged and the supernatant was used for further purification.

2.3. Anion exchange chromatography

The obtained supernatant was filtered with a 0.45 μ m filter and loaded on to a 5 mL anion-exchange chromatography column (QHP - Hiload HP, GE healthcare, Uppsala, Sweden) previously equilibrated in Washing Buffer (20 mM Na Phosphate pH 7.4; 0.015% (w/v) β -DDM) at a flow rate of 0.5 mL/min. After injection, washing was performed at a flow rate of 0.5 mL/min until the absorbance was stable to zero for 5 column volumes. Finally, the bound components were eluted in a 70 mL gradient of 0–100% Elution Buffer C (20 mM Na phosphate pH 7.4, 2 M NaCl).

2.4. Size Exclusion Chromatography

A pool of the peak fractions was subsequently subjected to Size Exclusion Chromatography (SEC). The protein sample was loaded on a gel filtration column (Superose 6 10/30 GL, GE Healthcare) pre-equilibrated with Washing Buffer (20 mM Phosphate Buffer pH 7.4; 0.015% (w/v) β -DDM). The molecular weight of complexes was estimated by plotting the elution volume of the main peaks versus the logarithm of the molecular weight of the standard proteins (Gel Filtration Standard, Biorad) using a polynomial regression curve fit - second order (Farci et al., 2016).

2.5. Polyacrylamide gel electrophoresis (PAGE)

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with a 10% (w/v) separating and a 4% (w/v) stacking gel was used as described in (Farci et al., 2016). Before loading, samples were subjected to denaturation by using Rotiload (Roth) or a home-made loading buffer (3.75 M Tris-HCl pH 6.8; 5% (w/v) SDS; 15% (v/v) β -mercaptoethanol; 30% (w/v) glycerol; 0.3% (w/v) bromophenol blue). In both cases, samples were boiled for 5' and subsequently centrifuged for 5'. Separation was performed at 100 V for 2 h and, after electrophoretic separation, gels were removed, washed in distilled water, and stained with Coomassie Brilliant Blue G250. Blue Native-Polyacrylamide Gel Electrophoresis (BN-PAGE) was carried out using 3–12% (w/v) continuous gradient gels, according to Haniewicz et al. (2015), and Farci et al. (2017). Samples were mixed 1:3 in native loading buffer (50 mM Bis-Tris pH7.2, 6 N HCl, 50 mM NaCl, 10% w/v Glycerol, 0.001% Ponceau S final concentrations). Electrophoresis was carried out at 205 V for 5 h at 4 °C.

2.6. Mass spectrometry analysis

Bands from the BN- and SDS-PAGE were excised, subjected to trypsin digestion and processed as reported in Farci et al. (2016, and Collu et al. (2017). Raw data were searched using MaxQuant (v. 1.5.3.30) with Andromeda (Cox et al., 2011) against a species specific database (Uniprot Capsicum Annum, 75,326 entries, 2018_07_13) with a database of common contaminants appended. The proteingroups.txt

table was then filtered to remove reverse entries, common contaminants and any proteins that did not meet a minimum Andromeda score of 40. Finally, for each dataset a relative scale of representativity was established defining an index of contribution (ratio) calculated between a given entry and the highest iBAQ in the dataset. A cutoff was applied excluding all the ratio with index below 0.01. Data are available via ProteomeXchange with identifier PXD013617.

2.7. Activity assay

Functional tests were performed promoting the backward reaction of the CCS. A mix of 38 μL in ratio 1:1 of the pure CCS (~ 0.05 mg/mL) and an excess of its product pool, the ~ 800 kDa carotenoid aggregate, was incubated at 20 °C. The reaction was promoted by adding 2 μL of NAD^+ 100 mM (Carl Roth, $\geq 98\%$) as a cofactor. The level of activity was estimated by recording the absorption at 340 nm, which is characteristic for NADH formation and typical when the backward reaction is taking place. All the reactions were performed at a controlled pH of 7.4 in Washing Buffer. Measurements were also performed at wavelengths between 400 and 600 nm, a region of the spectrum where carotenoids show their typical $S_0 \rightarrow S_2$ transitions related to the polyene organization (Miller, 1934). In this region a net increase of the absorbance was observed at a wavelength of 459 nm, providing an internal reference for the carotenoid synthesis. The enzyme activity was monitored for 45 min at intervals of 2.5 or 5 min.

3. Results

3.1. The total membranes fraction is constituted by two dominant proteins

Chromoplast membranes were isolated and solubilized using the mild detergent n-dodecyl- β -D-maltoside (β -DDM). The protein composition of the solubilized fraction was analysed by SDS-PAGE and the samples resolved into several faint bands with molecular weights spanning between 100 and 10 kDa (Fig. 1). Among this pattern of bands two highly expressed proteins carrying an apparent mass of about 50 kDa appeared to be more evident (Fig. 1). Solubilized membranes also appeared with an intense-brilliant red colour due to pigments associated to the membrane fraction (Fig. 1, inset).

3.2. A main protein, identified as the enzyme capsanthin/capsorubin synthase, occurs in concomitance with the protein annexin

Solubilized membranes were subjected to a step of anion-exchange chromatography, through which a main peak and a subsequent secondary one at higher ionic strength could be separated (Fig. 2). Interestingly, the isolated pool of fractions from the main peak appeared with a brilliant red colour (Fig. 2, left inset), suggesting the possible co-presence either of a pigmented cofactor, or of a substrate/product that might be related to some enzymatic activity associated with the proteins of the pool. Under denaturing electrophoresis conditions, the isolated pool of fractions from the main peak resolved in two dominant bands, the first with an apparent molecular weight of ~ 50 kDa and the second of ~ 35 kDa (Fig. 2, top inset). Small amounts of other minor components were also found (Fig. 2, top inset). The two main bands were analysed by MS, allowing the identification of the heavier band as the enzyme capsanthin/capsorubin synthase (CCS), and for the lighter one the protein annexin (Tables 1a and 1b, respectively). These two proteins represented the main components of their respective bands, while to each other they appeared as secondary component (Table 1), with few minor contaminants. The CCS is essential in the synthesis of both capsanthin and capsorubin from antheraxanthin and violaxanthin, respectively (Bouvier et al. 1994; Hirschberg, 2001; Li and Hui, 2013), and its presence is consistent with the pigmentation associated to the fractions of the main peak (Fig. 2, left inset).

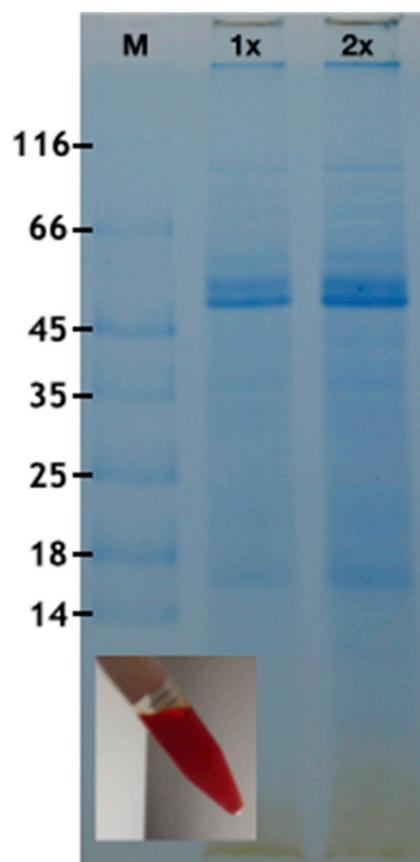


Fig. 1. Denaturing gel of solubilized membranes. The soluble chromoplast fraction analysed by SDS-PAGE resolved in a typical pattern of bands with two dominant proteins; lanes 1x and 2x indicate the relative amounts loaded, 3 and 6 μL of solubilized membranes (total protein concentration $\sim 1.50/2.0$ mg/mL), respectively; the lane M indicates the molecular marker; a typical pool of solubilized membranes carries a characteristic brilliant red colour (inset). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

3.3. The enzyme capsanthin/capsorubin synthase occurs as a trimer strictly associated with organised aggregates of carotenoids

In order to understand whether the two proteins were subunits of the same complex and to increase the purity of the main protein, the sample was further subjected to a SEC step. The sample resolved into two main peaks with an estimated mass of ~ 800 kDa and 167 kDa, respectively (Fig. 3). The first peak appeared to be intensively pigmented and did not show proteinaceous constituents when analysed by SDS-PAGE, suggesting that it is only constituted of pigments, most likely carotenoids (Fig. 3, inset). Considering the reproducible retention mass associated to this non-proteinaceous peak, these carotenoids may occur as highly organised aggregates, as extensively reported in other species (Billsten et al., 2005; Wang et al., 2012; Hempel et al., 2015; Ishigaki et al., 2017).

The second peak in SDS-PAGE consisted of a single band of ~ 50 kDa (Fig. 3) identified as the CCS by MS analysis (Table 2b; Sup. Fig. 1), showing that the SEC step efficiently separated the heavier protein from the lighter one (Fig. 3, inset), which appeared as a small perturbation in the baseline profile at high elution volumes (20 mL). This suggested that the main protein is organised *in vivo* as a homo-oligomer and that the lighter band may occur as a co-purified component or, perhaps, only lightly bound to the main protein. In this respect, given an estimated mass of ~ 167 kDa for the main SEC complex and a mass of ~ 50 kDa for its monomer (Fig. 3), it can be assumed that the protein occurs in form of homo-oligomeric trimers (Fig. 3).

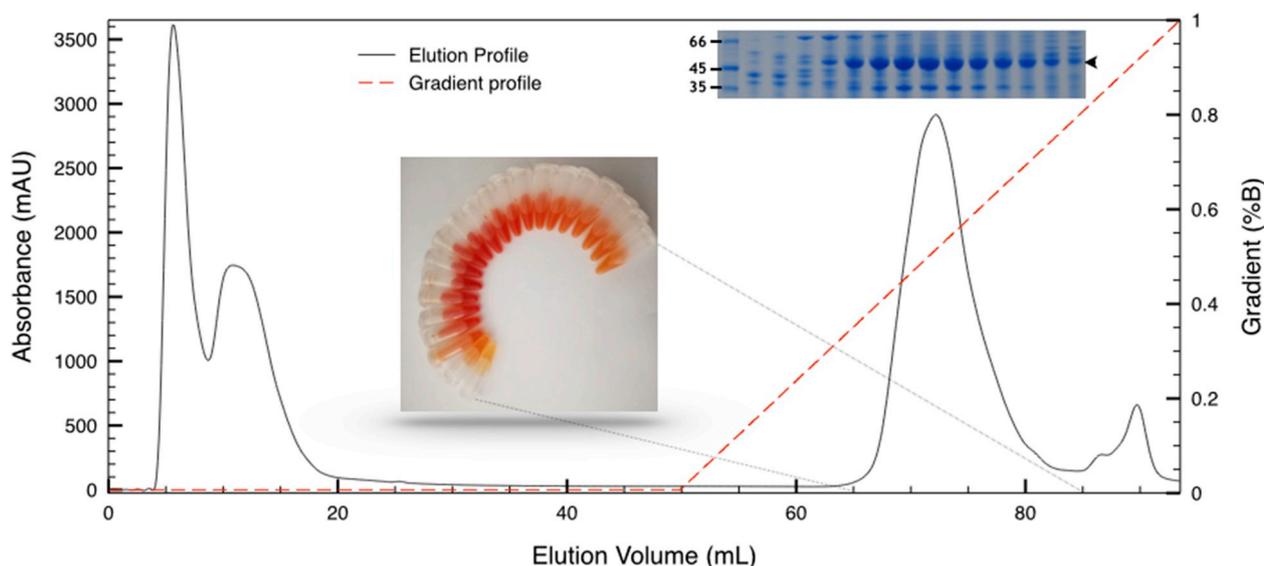


Fig. 2. Anion exchange chromatography of solubilized membranes. A step of Q Sepharose High Performance Anion Exchange Chromatography (QHP) was used to isolate the protein components present on the soluble fraction of chromoplast membranes. In the plot the dark solid line indicates the protein elution, while the dashed red line indicates the elution gradient; typically, a main peak followed by a less relevant one can be observed. The top inset shows the eluted fractions of the main peak analysed by SDS-PAGE; the black arrow shows the main protein isolated; molecular weights of the marker are also indicated. The left inset shows the characteristic pigmentation of the fractions associated to the main peak. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

As a further test, the oligomerization states associated to the CCS were assessed by BN-PAGES. In these experiments the protein pool clearly separated into two different bands (Sup. Fig. 1). The first band, as expected, was found to migrate at an apparent mass of ~170 kDa, while to the second one was associated an apparent mass of ~500 kDa. The absence of the larger oligomeric state in the SEC analysis suggests that it might represent a non-physiological higher oligomer. In both cases the protein identity was confirmed by the MS analysis performed on the native complexes (Tables 2a and 2b). The same analysis also enabled the possibility to determine whether other possible proteins, found in lower amounts, may transiently contribute to the oligomer's formation, providing some insights into the interactome network of the CCS (Table 2). While at this stage it cannot be stated to what extent other proteins are either co-purified or part of the CCS network, a physiological consistency of the data shown here is supported not only by the meaningful representativity of these proteins with respect to the CCS occurrence (Tables 2a and 2b), but also by the fact that all these proteins are involved in metabolic pathways of the fruit ripening that involves carotenoids (Tables 2a and 2b) (e.g. annexin and lipocalin protein - Wang et al., 2013; Pilbrow et al., 2014).

3.4. Both the organised aggregates and the capsanthin/capsorubin synthase bind carotenoids

The two pools isolated by SEC were subsequently subjected to room temperature UV-Vis absorption spectroscopy. Both samples showed a typical “three finger” spectrum characteristic for carotenoids (Fig. 4). Main absorption bands were found to be i) at 457 nm for both samples in buffer, ii) at 479 nm for the protein samples with a shift to 483 nm for the organised aggregates, and, finally, iii) at ~430 nm, which was evident only for the protein samples (Fig. 4). When extracted in ethanol, carotenoids from the same samples differed significantly with respect to the previous analysis. In particular, both shared a band at ~449 nm, which was evident for the extracts obtained from protein, but appeared only as a shoulder in extracts obtained from organised aggregates. Furthermore, the second band at 471 nm for the extracts obtained from protein samples appeared shifted to 476 nm for organised aggregates. In all cases, the third band was barely pronounced (Fig. 4).

From the features associated to the absorption spectrum and knowing that the isolated protein complex is the CCS, it can be assumed that the carotenoids present in the samples are either one of the enzyme substrates (antheraxanthin or violaxanthin) and/or one of the enzyme products (capsanthin or capsorubin). These experiments not only showed that the substrate/product is associated to the CCS samples, but also confirmed the carotenoid origin of the organised aggregates for the first SEC peak.

3.5. Organised carotenoids aggregates are accumulated during ripening

Several isolations were performed, beginning with fruits at different ripening stages. From these experiments, it emerged that while the ripening proceeds, the amount of organised carotenoids aggregates increases with respect to the amount of the CCS. This is evidenced by SEC runs normalised with respect to the CCS peak (Fig. 5). This approach not only demonstrated that the level of aggregates increases during the ripening, but also evidenced a positive trend of carotenoids aggregates accumulation (and CCS amounts) with respect to time (Fig. 5, inset). On the basis of these experiments, data were analysed by plotting the ripening stage vs the ratio between amount of organised carotenoids aggregates and CCS amounts, showing that during ripening an accumulation rate of pigments according to a polynomial growth is followed (Fig. 5, inset).

3.6. The enzyme capsanthin/capsorubin synthase is isolated in its functional form

Next, we asked whether the isolated CCS trimeric complex was on its active form and if the isolated carotenoids aggregates were truly products of this complex. Considering that the CCS catalyses a reversible reaction, to answer these questions we choose to perform an enzymatic assay using the isolated carotenoids aggregates as substrate, thus promoting the backward reaction of the enzyme. The aggregates isolated by SEC were added to the purified CCS sample in presence of NAD^+ . The backward reaction was monitored at the wavelength of 340 nm, a typical NADH absorption peak (Fig. 6). Consistently with the known reaction pathway of the CCS (Bouvier et al. 1994; Hirschberg, 2001; Mialoundama et al., 2010; Li and Hui, 2013), the addition of both

Table 1
Mass spectrometry analysis performed on the bands resolved by SDS-PAGE of the main Anion Exchange Chromatography (QHP) pool. In a) the main protein components identified by MS analysis on the ~50 kDa band are indicated, in b) the main protein components identified by MS analysis on the ~35 kDa band are indicated. Highlighted in bold are the entries related to the ripening process.

Protein ID	Metabolic pathway	Ripening-related process	Molecular Weight (kDa)	Unique sequence coverage (%)	Score	iBAQ SDS1 (value x 10 ⁺⁸)	Weighted presence ^a
Q2435_GCS_CAPAN chromoplasmic	carotenoids synthesis	veraison	56,658	75,3	323,310	7,09	1
J7H350_CAPAN chain	photosynthesis	chloroplast to chromoplast transition	52,92	48,2	323,310	0,45	0,063
A0A2G2ZU43_CAPAN reductase	photosynthesis	chloroplast to chromoplast transition	1675	30,2	79,120	0,0816	0,011
Protein ID	Metabolic pathway	Ripening-related process	Molecular Weight (kDa)	Unique sequence coverage (%)	Score	iBAQ SDS2 (value x 10 ⁺⁸)	Weighted presence ^a
Q9SB88_CAPAN Annexin	lipids degradation (peroxidation)	chloroplast to chromoplast transition	35,585	52,2	235,250	2,81	1
A0A2G2ZIA2_CAPAN	n.d.	n.d.	40,229	18,1	84,270	0,19	0,065
At1g06690	glycolysis	veraison	42,008	17,2	105,300	0,18	0,065
A0A2G3A4H8_CAPAN	oxidoreductase	chloroplast to chromoplast transition	40,407	24,6	44,447	0,16	0,056
Q9M4D2_CAPAN	carotenoids synthesis	veraison	56,658	75,3	323,310	0,15	0,052
Q2435_GCS_CAPAN chromoplasmic	n.d.	n.d.	36,322	3,5	6222	0,08	0,027
A0A1U8EK10_CAPAN	chromatin organization	veraison	12,556	8,1	6030	0,08	0,026
LOC107847185	carotenoid metabolism and sequestration	veraison and chloroplast to chromoplast transition	35,26	12,4	36,970	0,04	0,015
A0A2G2XV67_CAPAN	carotenoids synthesis and membrane lipids degradation (peroxidation)	veraison and chloroplast to chromoplast transition	58,514	41	303,810	0,04	0,015
Q42493_CAPAN	porphyrin-containing compound biosynthesis	chromoplast transition	40,906	5,5	12,610	0,04	0,015
A0A2G3A1U1_CAPAN	porphyrin-containing compound biosynthesis	chromoplast transition	44,841	4,5	18,408	0,04	0,013
A0A1U8GXF8_CAPAN	chromatin organization	veraison	15,406	5,9	6420	0,03	0,012
A0A2G2YR06_CAPAN	chromatin organization	veraison	11,425	21,4	11,724	0,03	0,010
Q6RUR1_H33_CAPAN	n.d.	n.d.	41,328	6,5	19,267	0,03	0,010
Q71V09_H4_CAPAN	myo-inositol biosynthesis	veraison	40,112	8	32,749	0,03	0,010
A0A2G3A299_CAPAN							
LOC107862232							
A0A2G3A6N6_CAPAN							

^a Weighted presence: iBAQ/the highest iBAQ values among the pool identified.

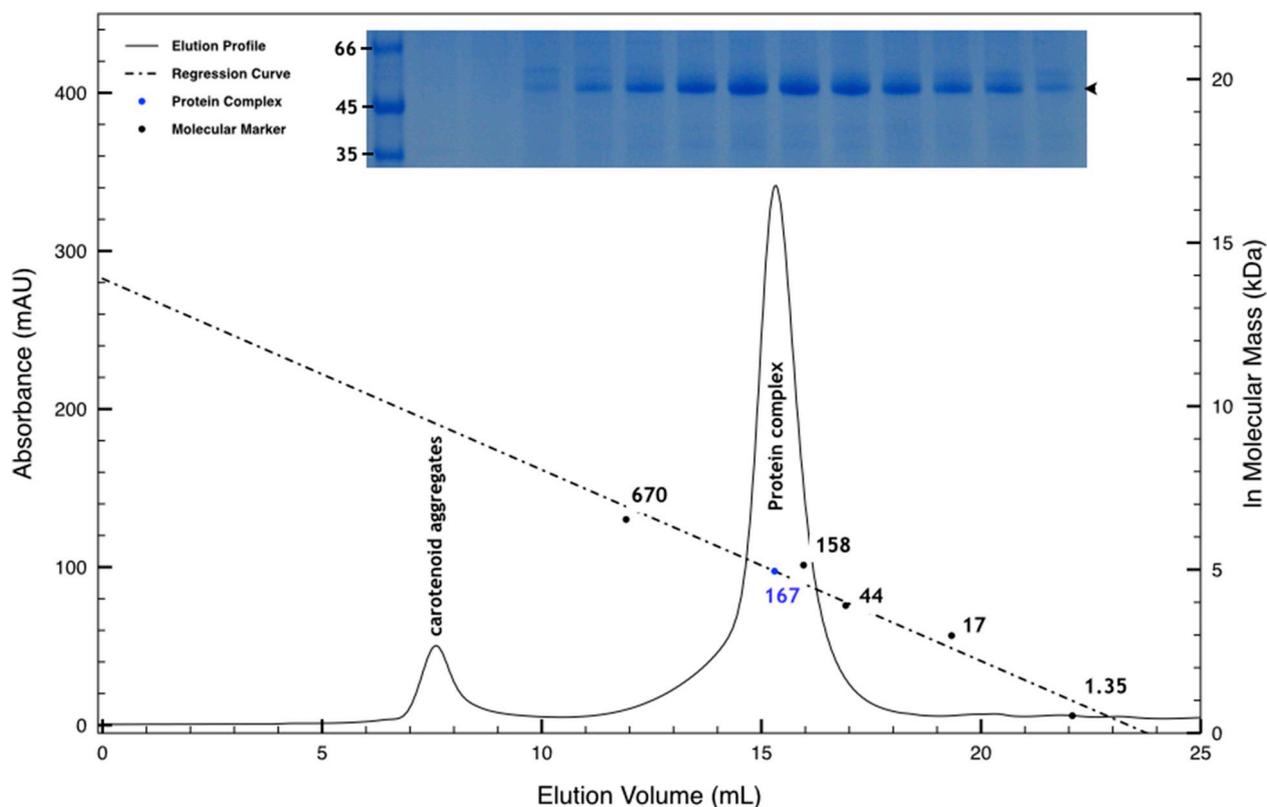


Fig. 3. Size Exclusion Chromatography of the isolated protein complex. SEC profile from the concentrated pool of the main anion exchange peak (black solid line). The mass of the protein complex (blue dot) was estimated on a Superose 6 column with respect to a regression curve (dash-dotted line) on the base of the molecular marker (black dots). The elution profile also shows a reproducible peak eluting in the void volume that was found to be non-proteinaceous and ascribable to free carotenoid aggregates. The inset shows a SDS-PAGE of the peaks separated by SEC; the black arrow indicates the main protein; molecular weights of the marker are also indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

the product and the oxidized cofactor (NAD^+) promoted the backward reaction that sees the synthesis of violaxanthin starting from capsorubin and the contemporary formation of NADH during the reaction.

As shown in Fig. 6, the peak of activity is reached at about 10 min, while are needed about 40 min in order to get back to zero and hence to the new equilibrium. Absorption changes at 459 nm, a wavelength localized into the region of the carotenoid spectrum associated to their typical $S_0 \rightarrow S_2$ transitions related to the polyene organization (Miller, 1934), were also observable (data not shown). At this wavelength a net increase of the absorbance was observed, providing an internal reference for the carotenoid synthesis concomitantly with the increase at 340 nm due to the NADH formation.

This activity assays not only proved the CCS functionality, but also suggested important hints about the origin of the carotenoid aggregates isolated by SEC. In fact, the presence of NAD(P)^+ as CCS cofactor is reported to be essential only for the reaction that brings from violaxanthin to capsorubin (Camara, 1980; Gómez-García and Ochoa-Alejo, 2013). During this reaction there is a consequent production of NADH, which can be used for the backward reaction from capsorubin to violaxanthin. On the contrary, neither NAD(P)^+ nor NAD(P)H affect the reaction from antheraxanthin to capsanthin (Camara and Moneger, 1981; Gómez-García and Ochoa-Alejo, 2013), thus, in this case only the internal FAD cofactor plays a role. Accordingly, a NADH production would not be observed in the case of the other backward reaction from capsanthin to antheraxanthin (Camara and Moneger, 1981; Gómez-García and Ochoa-Alejo, 2013).

4. Discussion

Chromoplasts are plant cell organelles that derive from a complex remodelling of the internal chloroplast membranes and are designed for

carotenoid's synthesis and accumulation. Extensively studied are chromoplasts from tomato fruits, which accumulate carotenoids mostly as lycopene crystalloids in membranaceous structures (Harris and Spurr, 1969), and from *C. annuum* fruits, which are characterized by a large amount of fibril-containing chromoplasts. In the latter case, carotenoids are known to be sequestered by a layer of polar lipids surrounded by the lipoprotein fibrillin (Deruère et al. 1994; Kilcrease et al., 2013). These storage structures are extremely important for promoting the carotenoids biosynthesis by removing the products from the media and avoiding the inhibition by retro-regulation mechanisms (Egea et al., 2010). As expected, the transition from chloroplast to chromoplast sees not only a remodelling of membranes associated to an intense recycle and export of molecules, but also the *ex novo* formation of the mentioned carotenoids storage structures in association with an increased expression rate of the enzymes involved in the carotenoid's biosynthesis (Bian et al., 2011). Among the plastids, chromoplasts and gerontoplasts share an important feature: their cells undergo a gradual process whereby they lose their autotrophy due to the lack of chloroplasts (Hall, 1977; Fisher et al., 2012; Solymosi and Keresztes, 2012). This sequence of events makes the cells exclusively dependent on mitochondria and their respiration. As a result, during this transition, the cells are evidently subjected to a stress condition resulting from the metabolic imbalance and the consequent compromised homeostasis. These events are also marked by significant oxidative stress, which is associated with the formation of both nitrogen and oxygen reactive species. During differentiation, in chromoplasts and gerontoplasts, the removal and export of components is normally associated with the neosynthesis of molecules with antioxidative properties (carotenoids, flavonoids, and derivatives of phenylpropanoids) and to the import of molecules with antibiotic function. These aspects give an indication of the extent to which these sites are subjected to oxidative stress and, thus, represent

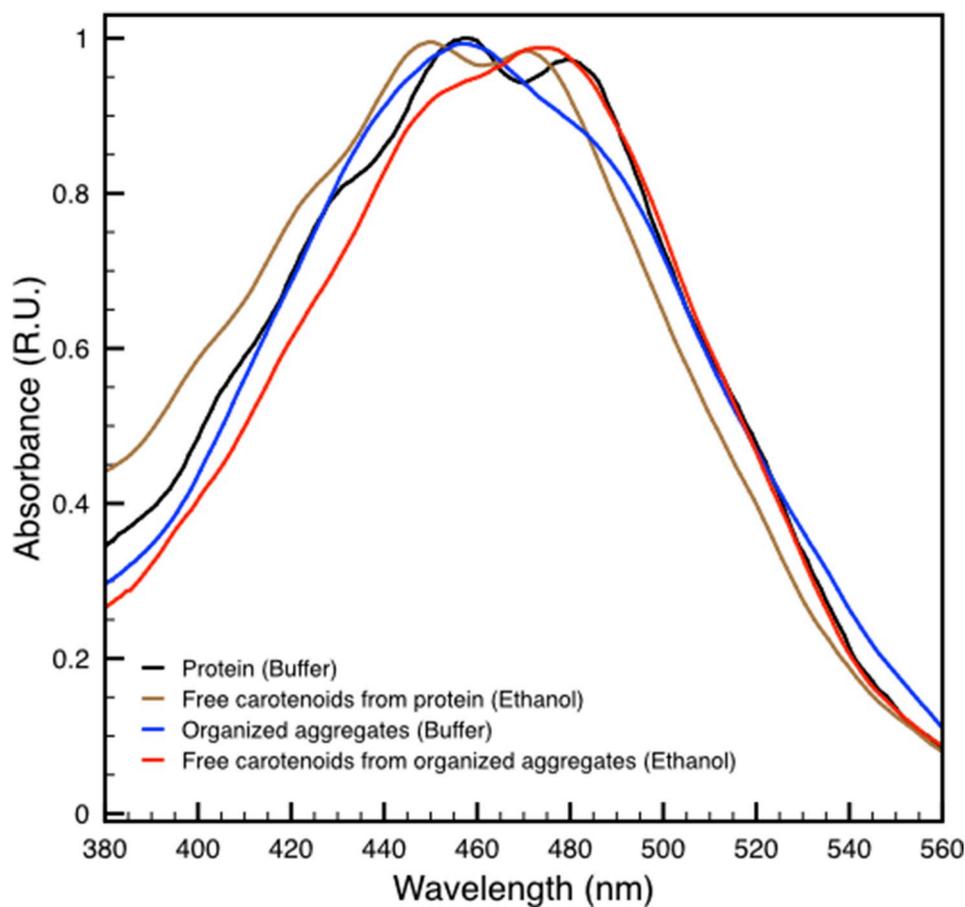


Fig. 4. Absorption spectroscopy analysis of the isolated protein complex and extracted carotenoid. Room temperature absorption spectra of the CCS complex (black line) and of the organised aggregates (blue line) in buffer as resolved by SEC. Solvent extract from the same samples, protein (brown line) and organised aggregates (red line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

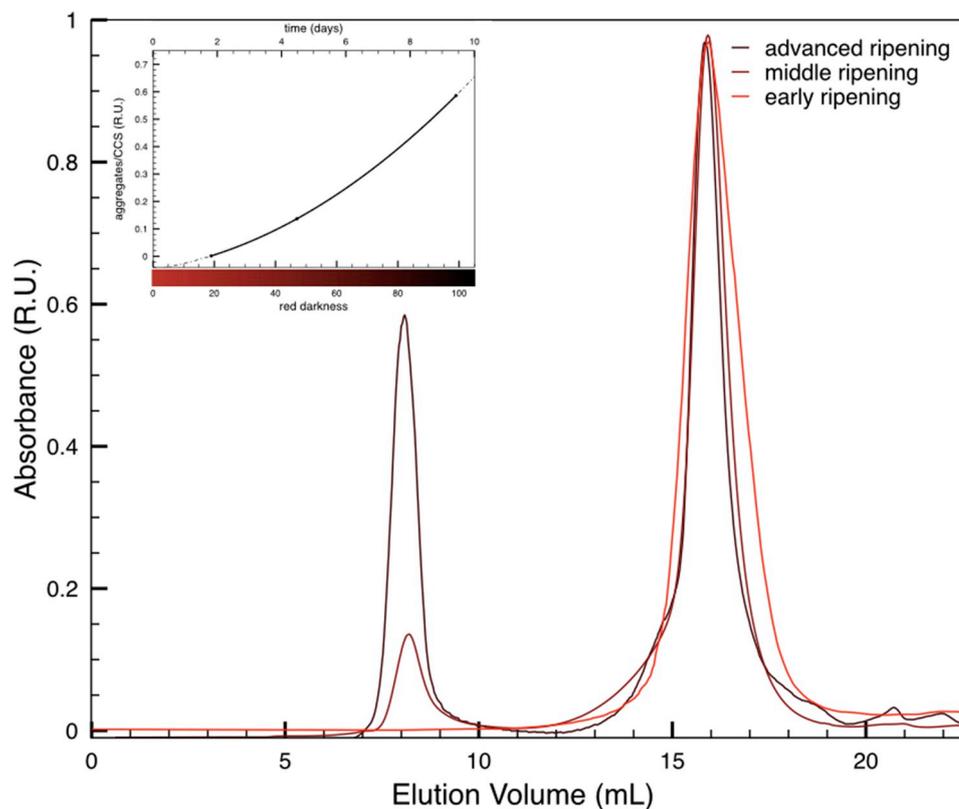


Fig. 5. Comparison between the amounts of organised carotenoids aggregates at different ripening stages. SEC runs on samples isolated from three different ripening stages early (light red), middle (red), and advanced (dark red). The runs are normalised with respect to the CCS peak. The inset shows the accumulation trend of aggregates with respect to time and level of pigmentation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).

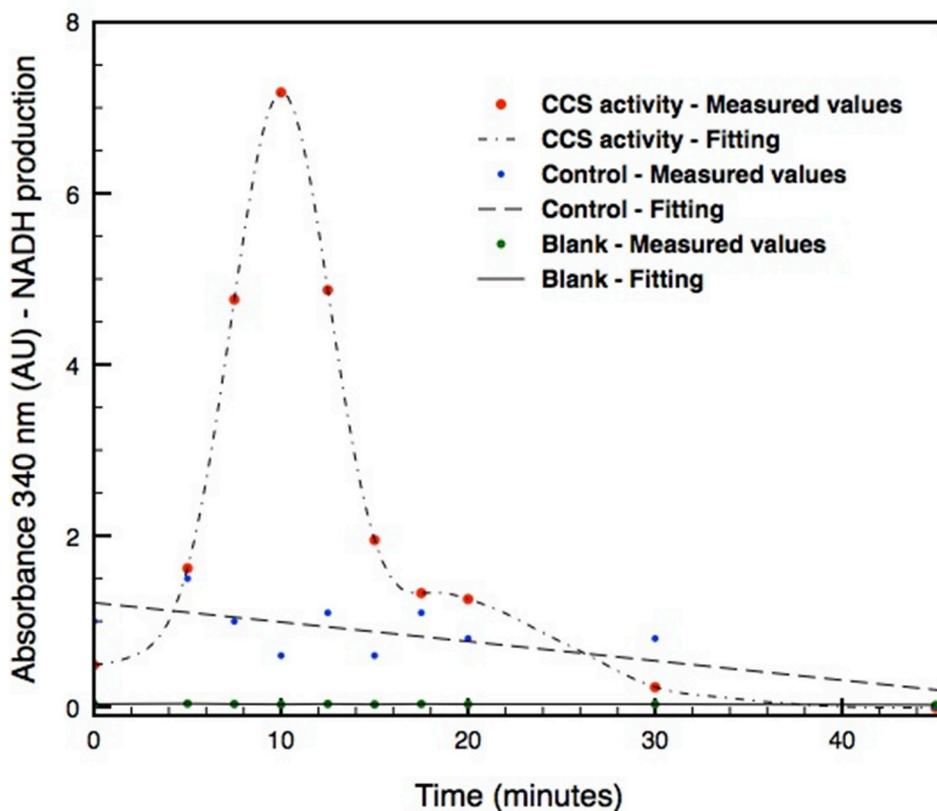


Fig. 6. CCS functional assay. Functional tests were performed promoting the backward reaction of the CCS from capsorubin to violaxanthin. The pure CCS was incubated at 20 °C in presence of its product and NAD⁺ as a cofactor. The activity was estimated by recording the absorption at 340 nm characteristic of the NADH formation. The enzyme activity was monitored for 45 min. Colour legend is shown in the figure's inset (top right).

places where protective mechanisms are required to be activated in order to maintain cell homeostasis. Accordingly, the ripening process itself has important similarities with the process of senescence in leaves. This observation is not surprising when considering that they share the common ontological root that is the apical stem (Spurr and Harris 1968; Seymour et al., 1993; Solymosi and Keresztes, 2012; Batista-Silva et al., 2018; Sun et al., 2018; Li et al., 2017; Alos et al., 2019). When viewed in this context, chromoplasts in ripening fruits and gerontoplasts in senescent leaves share more than just a chromatic variation from green to red, yellow, or orange. They share the primary need to protect the organ from early damage induced by oxidation, an event that assumes even more relevance in fruits, where the ripening proceeds in parallel with maturation of seeds (Solymosi and Keresztes, 2012; Osorio et al., 2013; Kumar et al., 2016). Accordingly, the colourfulness of fruits, important for their dispersion and seed diffusion, may be just a secondary and extrinsic reason, whilst the protection of the organ from the oxidative events occurring during the ripening may be seen as the primary and intrinsic reason.

The synthesis and the accumulation of carotenoids, capsanthin and capsorubin in the case of *Capsicum spp.*, represent the main event during fruits veraison (Valadon and Mummery 1976; Schweiggert et al. 2005; Ha et al., 2007). Because of the beneficial effects of these carotenoids on human health as nutraceutical/pharmaceutical compounds (Gómez-García and Ochoa-Alejo, 2013), omics studies have deeply investigated the biodiversity into the carotenoids biochemical pathways of *C. annuum*. These studies allowed to unveil the differences in fruit colours and to characterize specific genotypes/phenotypes differences with the aim to select cultivars carrying a high-capsanthin/capsorubin content (Jeknić et al., 2012; Jeong et al., 2019; Konishi et al., 2019). Consequently, also the enzymes responsible for their biosynthesis, in this case the capsanthin/capsorubin synthase (CCS), assume a pivotal role. In order to gain insight into the role of the carotenoids-CCS system on ripening, we have performed a transversal study aimed at understanding the CCS's properties and the relationships with its products

while ripening is proceeding. Moreover, we have also tried to identify other proteins involved in this complex interactome network.

Initially, the CCS was found to be present as a monomer on the chromoplast membrane fraction isolated from *C. annuum* fruits (Bouvier et al. 1994). In this study, starting from *C. annuum* fruits, we have performed the isolation of the CCS from the membranes fraction by following a very mild procedure (Fig. 1). The SDS-PAGE analysis on these samples unexpectedly showed that, among the dominant components, the fibrillin was present in low amount, even though this protein was considered one of the main components in *C. annuum* chromoplasts (Siddique et al., 2006). Because of its function, fibrillin is also considered intimately related with the chromoplast membrane, thus, its limited presence seems to just highlight a representativity lower than previously expected. In support to this conclusion, the MS analysis performed on native samples identified significant amounts of both subunits of the reference enzyme Rubisco, characteristic for plastids, with respect to fibrillin (Sup. Fig. 1; Table 2). In this contest, the present study provides insights with respect to the strategies of carotenoids accumulation in *C. annuum* chromoplasts. In particular, the performed proteomic analysis supports previous genomic and transcriptomic studies on these poorly-understood but important ultra-structural sites deputed to carotenoids storage (Berry et al., 2019).

This mild procedure allowed to isolate the CCS under *quasi-native* conditions, identifying it as organised into trimers, as confirmed by SEC and BN-PAGE (Fig. 3; Sup. Fig. 1). Furthermore, BN-PAGE also identified a much heavier band of CCS, the identity of which was subsequently confirmed by MS and found to be equivalent to a CCS nonameric form, for which, at the moment, a physiological relevance cannot be ruled out. Independently from the dominant oligomeric state *in vivo* (trimers or nonamers or both), the multimeric organization is likely to have a much greater physiological relevance than the monomeric one and is a common characteristic feature of enzymes that are called to process substrates at a high rate, an essential requirement for fruits' veraison (Sanz et al. 2002; Fraser and Bramley 2004).

The two-dimensional chromatography approach, anionic exchange followed by size exclusion, combined with MS analysis through SDS-PAGES and BN-PAGES not only allowed the investigation of the oligomeric state of the CCS, but also the identification of other proteins involved in the CCS network. In particular, after the first chromatographic step, a co-purified protein was also isolated, which the MS analysis excluded as being a degradation product of the CCS and identified it as annexin 1. Annexins are extensively reported to be essential in the transition from chloroplast to chromoplast and are most likely involved in the CCS network (Wang et al., 2013). Since these two proteins represented the main components of their respective bands, while to each other appeared as secondary components (Fig. 2, inset; Table 1), a possible mild interaction between the CCS complex and annexin 1 cannot be excluded. Moreover, associated to the bands from samples isolated by the first chromatographic step are also other minor components, which, interestingly, are also involved in carotenoids biosynthesis, fruit ripening, and chromoplast differentiation (Table 1; Table 2). Similar observations are also valid for the minor constituents found to be associated to the CCS bands in the BN-PAGE of the second main peak obtained in SEC (Sup. Fig. 1; Table 2).

Interestingly, the CCS fraction isolated in purity by SEC (Fig. 3) led to samples evidently pigmented and the protein pool obtained after both chromatographic steps did not show any loss of pigments as flow-through during concentration. These observations strongly support the idea that the CCS retains carotenoids *in vivo*. On this basis, the spectral analysis on the samples excluded the presence of the possible substrates, antheraxanthin and violaxanthin, and confirmed the presence of the two possible products, capsanthin and capsorubin (Fig. 4). In contrast, only the typical absorption bands of capsorubin could be seen in association with organised carotenoids aggregates, as also confirmed by the functionality assay of the CCS (see paragraph 3.6 and related Fig. 6). Organised carotenoids aggregates were identified and separated in reproducible manner with a conserved mass by SEC (Fig. 3). Organised aggregates were completely unexpected for *C. annuum*, even though similar structures were extensively described for lycopene in *S. lycopersicum* fruits (Harris and Spurr, 1969; Ishigaki et al., 2017). Moreover, the relatively low representativity of the protein fibrillin associated to these structures (Fig. 1; Table 1; Table 2) also allow to hypothesize a slightly different chromoplast organization in *C. annuum* with respect to the general vision where fibrillin is seen as one of the main proteins of chromoplasts.

Finally, it was extremely interesting to find a strict relationship between the organised aggregates and the degree of ripening (Fig. 5), suggesting that these structures are the main units of carotenoids' accumulation in *C. annuum* fruits.

Results shown here are obtained by following a *quasi-native* approach, that provided insights into the CCS's properties, characterized by two important features: i) the presence of higher oligomeric states such as trimers and nonamers and the absence of monomers; ii) the specific retention of capsorubin and capsanthin in its structure. These observations allowed further investigation of two important aspects related to *C. annuum* ripening and its chromoplasts' organization: i) the pivotal role played by the organised aggregates accumulated during ripening, which is similar to that observed in *S. lycopersicum*, and ii) the unexpected low representativity of the fibrillin protein considered, so far, one of the main components in the chromoplast proteome of this species. The presented results extend our current understanding of the organization and functionality of CCS in *C. annuum* chromoplasts and, similarly to what has been observed in *S. lycopersicum*, carotenoids appear as organised aggregates rather than structures that are coordinated by a proteinaceous matrix. While further studies are still ongoing, the present findings suggest a preliminary, but significant *trait d'union* between *C. annuum* and *S. lycopersicum* with respect to their chromoplasts functionality and dynamics during ripening.

Author contributions

DF conceived, designed, and coordinated the study, carried out the membranes preparation, protein isolation, biochemical and bioinformatic studies, and drafted the manuscript. DP participated in designing the study, biochemical and bioinformatic studies, and drafting the manuscript. EC participated in the membranes preparation, protein isolation and biochemical studies, and helped in drafting the manuscript. GG helped in the bioinformatic studies and drafting the manuscript. JK performed the MS analysis and helped in drafting the manuscript. HK helped in drafting the manuscript.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaíno et al., 2016; Deutsch et al., 2017) with the dataset identifier PXD013617.

Additional information

Authors declare no competing final interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.09.007>.

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