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RESEARCH PAPER

Characteristics of the tomato chromoplast revealed by proteomic analysis

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Abstract

Chromoplasts are non-photosynthetic specialized plastids that are important in ripening tomato fruit (*Solanum lycopersicum*) since, among other functions, they are the site of accumulation of coloured compounds. Analysis of the proteome of red fruit chromoplasts revealed the presence of 988 proteins corresponding to 802 *Arabidopsis* unigenes, among which 209 had not been listed so far in plastidial databanks. These data revealed several features of the chromoplast. Proteins of lipid metabolism and trafficking were well represented, including all the proteins of the lipoxygenase pathway required for the synthesis of lipid-derived aroma volatiles. Proteins involved in starch synthesis co-existed with several starch-degrading proteins and starch excess proteins. Chromoplasts lacked proteins of the chlorophyll biosynthesis branch and contained proteins involved in chlorophyll degradation. None of the proteins involved in the thylakoid transport machinery were discovered. Surprisingly, chromoplasts contain the entire set of Calvin cycle proteins including Rubisco, as well as the oxidative pentose phosphate pathway (OxPPP). The present proteomic analysis, combined with available physiological data, provides new insights into the metabolic characteristics of the tomato chromoplast and enriches our knowledge of non-photosynthetic plastids.

Key words: Calvin cycle, carbohydrate metabolism, chromoplast proteome, lipid metabolism, oxidative pentose phosphate pathway, protein import, redox proteins, terpenoid metabolism, tomato, vitamins biosynthesis.

Introduction

Fruit ripening involves a series of biochemical and physiological events resulting in organoleptic changes in texture, aroma, and colour. In many fruit one of the most important and more visible changes corresponds to the loss of chlorophyll and the synthesis of coloured compounds such as carotenoids. Carotenoids accumulate in chromoplasts that are non-photosynthetic plastids often present in flowers and fruit and also occasionally found in roots and leaves. In both flowers and fruit, they serve the reproduction strategy of the plant by attracting pollinators and animals that disperse the seeds. In tomato, which is widely used as a model fruit, it is not clear whether chromoplast

differentiation is a consequence of the ripening process or whether chromoplasts play a role in the onset of the ripening process. It is well known that, in climacteric fruit, the ripening process is triggered by the plant hormone ethylene (Lelièvre *et al.*, 1997; Giovannoni, 2001) and fruit physiologists have contributed to the elucidation of the mechanisms governing the mode of action of ethylene and the accumulation of metabolites responsible for important quality attributes (e.g. aromas, vitamins, and antioxidants). In recent years, a number of genes and proteins involved in the fruit ripening process have been isolated through the implementation of modern genomics (Moore *et al.*, 2002) and proteomics (Faurobert *et al.*, 2007). However, little attention has been paid to the mechanisms of fruit ripening at the subcellular level. For instance, the detailed functioning of chromoplasts are not well understood despite their crucial role in the generation of major metabolites that are essential for the sensory and nutritional quality of fruit. A combination of experimental and bioinformatics data have estimated the size of the plastid proteome to be around 2700 proteins, amongst which more than 95% are imported (Soll, 2002; Millar *et al.*, 2006). The sequencing of the tomato chloroplast genome established that it contains 114 genes (Kahlau *et al.*, 2006) and that the differentiation of the chromoplast does not involve rearrangements of the plastid genome (Hunt *et al.*, 1986). Therefore, knowledge of the plastidial genome provides little informatiion about the proteins that reside in the chromoplast and that underlie the wide variety of metabolic and regulatory events associated with this organelle. Major programmes devoted to the generation of ESTs and to the sequencing of the genome have been initiated with tomato as a model plant, but the accumulation of data on global gene expression and on genome sequences remains of limited value in understanding the function of chromoplasts. In addition, these sequencing programmes can address neither the post-translational protein modifications nor the subcellular localization of the biosynthetic pathways. For these reasons, high-throughput proteomics associated with bioinformatics represents the most appropriate strategy towards identifying the protein components of the chromoplast and hence uncovering the multiple functions of the organelle. Comprehensive proteome information is expected to bring new insights into processes such as intracellular protein sorting as well as biochemical and signalling pathways. To date, the most important progress in relation to the plastid proteome has been made for chloroplasts (Kleffmann *et al.*, 2004; Zybailov *et al.*, 2008) and this analysis includes suborganelle protein localization for the thylakoid and lumen, (Peltier *et al.*, 2002; Schubert *et al.*, 2002), the stroma (Peltier *et al.*, 2006), the envelope (Ferro *et al.*, 2003), and plastoglobules (Ytterberg *et al.*, 2006). Advances have also been made in protein targeting mechanisms (Jarvis, 2008; Zybailov *et al.*, 2008). The proteomes of heterotrophic plastid types have been studied less extensively and are restricted to rice etioplasts (von Zychlinski *et al.*, 2005), wheat amylopasts (Andon *et al.*, 2002; Balmer *et al.*, 2006) and tobacco proplastids (Baginsky *et al.*, 2004). An analysis of the bell pepper chromoplast identified 151 proteins using MS/MS tandem mass spectrometry (Siddique *et al.*, 2006). Protein profiling of plastoglobules from pepper fruit chromoplasts and from *Arabidopsis* leaf chloroplast has also been performed, yielding around 20 proteins (Ytterberg *et al.*, 2006). In the present work, chromoplasts have been isolated from ripe tomato fruit and the soluble and insoluble protein fractions sequenced using LC-MS/MS LTQ-Orbitrap technology. This proteomic study substantially enlarges the number of chromoplastic proteins identified so far and provides new information on metabolic and regulatory networks in heterotrophic chromoplasts.

Materials and methods

Isolation of tomato chromoplasts

Approximately 300 g of tomato fruits (*Solanum lycopersicum* cv. MicroTom) were picked 10 d after breaker. The seeds and the gel were eliminated and the pericarp was cut in small pieces. The pieces of pericarp were rinsed twice in ice-cold extraction buffer (250 mM HEPES, 330 mM sorbitol, 0.5 M EDTA, and 5 mM β-mercaptoethanol pH 7.6). The whole suspension was then put in a cold Waring Blendor and blended by a short pulse at minimum speed. After filtering through two layers of gauze and 60 μm nylon net, the filtrate was centrifuged at 4 °C, 4000 rpm for 5 min, the supernatant discarded and the pellet recovered in 100 μl of extraction buffer. The pellet was the loaded onto a gradient made of three layers of 0.5 M, 0.9 M, and 1.45 M sucrose and then centrifuged 45 min at 4 °C at 62 000 *g*. Western blot and microscopic observations indicated that intact chromoplasts were located at the interface between the 0.9 M and 1.45 M sucrose layers.

Analysis of chlorophyll, carotenoids and tocopherols

The content in carotenoids, chlorophyll, and tocopherols of tomatoes at breaker +10 d was evaluated as described by Fraser *et al.* (2000).

Western blot analysis

In order to assess the degree of enrichment of the chromoplast fraction, Western blot analysis was performed using polyclonal antibodies at the appropriate dilution against chloroplastic photosystem II D1 protein (psbA/D1, 32 kD, at 1:10 000 dilution) and Rubisco large subunit (RubcL, 53 kDa, 1:50 000), cytosolic sucrose phosphate synthase (SPS, 120 kDa, 1:1000), mitochondrial voltage-dependent amino-selective channel protein 1 (Vdac1, 29 kDa, 1:1000), and vacuolar ATPase (V-ATPase, 26–27 kDa, 1:5000) from Agrisera® and cell-wall proteins, polygalacturonase (PG, 41-43 kDa, 1:5000) and pectin methyl esterase (PME, 31 kDa, 1:5000) generated by us from recombinant proteins corresponding to X77231 and X95991 cDNA, respectively. Total fruit proteins were extracted from fruit harvested at 10 d after breaker and ground in liquid nitrogen according to Campbell *et al.* (2003). Fruit and chloroplast proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare®), treated with blocking TTBS buffer [20 mM TRIS, 137 mM NaCl, 0.1% (v/v) Tween-20, pH 7.6, containing 2% (w/v) of ECL Advancing Blocking®, and subsequently incubated for 1 h with polyclonal antibodies diluted as indicated above in TTBS. Detection was performed with a peroxidase labelled anti-rabbit antibody (GE Healthcare®), diluted 1:50 000 in TTBS, and the membranes were developed using the GE Healthcare® Kit (ECL Advancing Western® blotting detection reagents). Western blot were made in duplicate from two chromoplasts isolations.

Fractionation of proteins

In order to increase the access to low-abundant proteins and therefore improve the efficiency of the proteomic analysis, chromoplasts were subfractionated into so-called soluble and insoluble factions. Chromoplasts of the 0.9–1.45 M sucrose interface were broken by osmotic shock adding 1:1 (v/v) 1 M HEPES buffer complemented with 2 mM DTT, followed by freeze/thawing and homogenization in a Potter-Elvehjem tissue grinder. The soluble fraction was obtained by two consecutive ultracentrifugations of the chromoplast extract at 100 000 *g* for 1 h at 4 °C. The two supernatants were mixed and precipitated overnight in methanol 1:6 (v/v) at –20 °C, then centrifuged at 16 000 *g*, for 30 min at 4 °C and the precipitate incubated for 2 h at room temperature in 4× Laemmli buffer [250 mM TRIS-HCl, pH 6.8, 40% glycerol (v/v), 8% SDS (w:v), 0.01% bromophenol blue (w/v)]. The pellet corresponding to the insoluble fraction was incubated in 4× Laemmli buffer overnight at room temperature. Proteins were quantified according to Bradford after TCA precipitation and resolubilization in 0.1 N NaOH.

SDS-PAGE

Samples (around 50 µg proteins) of soluble and insoluble fractions were boiled in SDS-sample buffer and then subjected to SDS-PAGE in 12% (w/v) polyacrylamide gel. After electrophoresis proteins were stained with PageBlue™ Protein Staining Solution (Fermentas).

LC-MS/MS as analytical method for the identification of chromoplast proteins

Each lane (soluble and insoluble fractions) from 1-D gel electrophoresis separation was cut into 15 homogenous slices that were washed twice in 100 mM ammonium bicarbonate/acetonitrile (1:1 v/v), 15 min at 37 °C. Proteins were digested by incubating each gel slice with 0.5 µg of modified sequencing grade trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate, overnight at 37 °C. The resulting peptides were extracted following established protocols (Wilm *et al.*, 1996) and final solution was dried in a Speed-vac.

The trypsin digests were reconstituted in 18 µl of 5% acetonitrile and 0.05% trifluoroacetic acid and then 5 µl were analysed by nanoLC-MS/MS using an Ultimate 3000 system (Dionex, Amsterdam, the Netherlands) coupled to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The peptide mixture was loaded on a C18 precolumn (300 µm ID×15 cm PepMap C18, Dionex) equilibrated in 95% solvent A (5% acetonitrile, 0.2% formic acid) and 5% solvent B (80% acetonitrile, 0.2% formic acid). Peptides were eluted using a 5–50% gradient of solvent B during 80 min at a 300 nl min^{-1} flow rate. Data were acquired with Xcalibur (LTQ Orbitrap Software version 2.2, Thermo Fisher Scientific). The mass spectrometer was operated in the data-dependent mode and was externally calibrated. Survey MS scans were acquired in the orbitrap on the 300–2000 *m/z* range with the resolution set to a value of 60 000 at *m/z* 400. Up to five of the most intense multiple charged ions (2+, 3+, and 4+) per scan were CID fragmented in the linear ion trap. A dynamic exclusion window was applied within 60 s. All tandem mass spectra were collected using normalized collision energy of 35%, an isolation window of 4 *m/z*, and 1 µscan. Other instrumental parameters included maximum injection times and automatic gain control targets of 250 ms and 500 000 ions for the FTMS, and 100 ms and 10 000 ions for LTQ MS/MS, respectively.

Database search and data analysis

Data were analysed using Xcalibur software (version 2.0.6, Thermo Fisher Scientific) and MS/MS centroid peak lists were generated using the extract_msn.exe executable (Thermo Fisher Scientific) integrated into the Mascot Daemon software (Mascot version 2.2.03, Matrix Sciences). Dynamic exclusion was employed within 60 s to prevent repetitive selection of the same peptide. The following parameters were set to create peak lists: parent ions in the mass range 400-4 500, no grouping of MS/MS scans, and threshold at 1000. A peaklist was created for each fraction (i.e. each gel slice) analysed and individual Mascot searches were performed for each fraction. Data were searched against the EST-tomato SGN database (2006-07-05) containing 208974 sequences and 59634226 residues (http://www.sgn.cornell.edu/). Mass tolerances in MS and MS/MS were set to 5 ppm and 0.8 Da, respectively, and the instrument setting was specified as 'ESI Trap'. Trypsin was designated as the protease (specificity set for cleavage after K or R), and one missing cleavage was allowed. Oxidation of methionine and deamidation of asparagine and glutamine were searched as variable modifications, no fixed modification was set. Mascot results were parsed with the home-made and developed software MFPaQ version 4.0 (Mascot File Parsing and Quantification) (Bouyssié *et al.*, 2007). Protein hits were automatically validated if they were identified with at least either: (i) one top ranking peptide with a Mascot score of more than 36.7 (for 36 proteins corresponding to this situation the spectrum of fragmentation is given in Supplementary Fig. S3 at *JXB* online); (ii) two top ranking peptides each with a Mascot score of more than 26.3; or (iii) three top ranking peptides each with a Mascot score of more than 22.9. To evaluate false positive rates, all the initial database searches were performed using the 'decoy' option of Mascot, i.e. the data were searched against a combined database containing the real specified protein sequences (target database, EST-tomato SGN database) and the corresponding reversed protein sequences (decoy database). MFPaQ used the same criteria to validate decoy and target hits, calculated the False Discovery Rate (FDR=number of validated decoy hits/(number of validated target hits+number of validated decoy hits)×100) for each gel slice analysed, and made the average of FDR for all slices belonging to the same gel lane (i.e. to the same sample). FDRs were below 1.6%. From all the validated result files corresponding to the fractions of a 1D gel lane, MFPaQ was used to generate, a unique non-redundant list of proteins that were identified and characterized by homology-based comparisons with the *Arabidopsis* database (TAIR8).

Database comparative proteomics, targeting predictions and functional classification

Proteins description were performed using annotations associated with each protein entry and through homology-based comparisons with the TAIR8 protein database (http://www.arabidopsis.org/) using BasicLocal Alignment Search Tool BLASTX (Altschul *et al.*, 1990) with an e-value cut-off of 1e-5 to avoid false positives, and linked. MapMan Bins were used for functional assignments (http://mapman.mpimp-golm.mpg.de/). The protein list was compared to three plastidial or subcellular localization databases: Plprot (Kleffmann *et al.*, 2006), PPDB (Sun *et al.*, 2008), and SUBA (Heazlewood *et al.*, 2007). Predictions of subcellular localization were undertaken using TargetP (Emanuelsson *et al.*, 2000; http://www.cbs.dtu.dk/services/TargetP/), Predotar version 0.5 (http://www.inra.fr/predotar/) and iPSORT (http://hc.ims.u-tokyo.ac.jp/iPSORT/). Predictions were made on the basis of tomato proteins when harbouring an N-terminal sequence. Otherwise predictions were made using *Arabidopsis* homologues. The homology search with plastidial proteins from AT, tobacco, rice, wheat, and chromoplastic proteins from pepper was determined by homology-based comparisons with the TAIR8 protein database (http://www.arabidopsis.org/). Sequence data arise from von Zyklinski *et al.* (2005) for rice etioplast, Baginski *et al.* (2004) for tobacco proplastids, Siddique *et al.* (2006) for pepper chromoplasts, Zybailov *et al.* (2008) for *Arabidopsis* chloroplasts, and Balmer *et al.* (2006) for wheat amyloplasts.

Results and discussion

Isolation of chromoplasts from red tomato fruit

The pellet of chromoplasts recovered as described in the Materials and methods was loaded onto a discontinuous gradient comprising 0.5, 0.9, and 1.45 M sucrose (Fig. 1A). Plastidial photosystem II D1 protein (psbA/D1) and mitochondrial voltage-dependent amino-selective channel protein 1 (Vdac1) marker proteins were detected by Western blotting (Fig. 1B). The presence of several bands cross-reacting with the anti-psbA/D1 antibodies may correspond to various forms of the protein during its processing and/or possible psbA/D1 complexes with other membrane proteins. The psbA/D1 protein is membrane-embedded in the large photosystem II complex (Campbell *et al.*, 2003). In the present proteomic analysis, it was only found in the insoluble fraction (see Supplementary Table S2 at *JXB* online). The layer of plastids at the 0.9/1.45 M interface was devoid of mitochondrial contamination and was assessed for purity using polyclonal antibodies against marker proteins of different cell compartments. As expected, proteins isolated from chromoplasts reacted with the anti RbcL antibodies (Fig. 1C). Proteins predicted to be located in the vacuole, cell wall, cytosol, and mitochondria could not be detected. Interestingly, the antibodies were able to detect all these marker proteins in total protein extracts of breaker+10 tomato fruit (Fig. 1C). These data indicate that the chromoplast preparation used for the subsequent proteomics analysis was of high purity.

Curation of isolated proteins by comparing with plastid databanks and by using predictors of subcellular localization

Western blot data indicated that there was little contamination. However, proteomic analysis revealed proteins that had not yet been annotated as plastidial and were absent from plastid databanks. We therefore curated the list of proteins by comparing with three plastid databanks (SUBA, PPDB, and PlProt) and by using three targeting preditors (Target P, Ipsort, and Predotar). The final list, comprising 988 tomato unigenes corresponding to 802 *Arabidopsis* unigenes is given in Supplementary Table S1 at *JXB* online. Amongst the 988 proteins, 360 were found in the so-called 'soluble' fraction extracted with the HEPES-DTT buffer, 170 in the so-called 'insoluble' fraction solubilized with the Laemmli-SDS buffer, and there were 458 in both fractions.

Figure 2 shows that 765, 506, and 332 chromoplast proteins corresponding to tomato unigenes were annotated in the SUBA, PlProt, and PPDB libraries, respectively. However, 209 proteins revealed by proteomic analysis were not in the databanks, but were predicted as being plastidial by at least one of the three targeting predictors. They can therefore be considered as novel plastidial proteins and have been overlined in Supplementary Table S1 at *JXB* online.

When comparing the tomato chromoplast proteome identified here with the proteome of other plastids on the basis of unique AT proteins (Fig. 3) it appears that the number of plastidial proteins identified in the present study (988) is of the same order of magnitude as the *Arabidopsis* chloroplast proteome (1280), but higher than the proteome

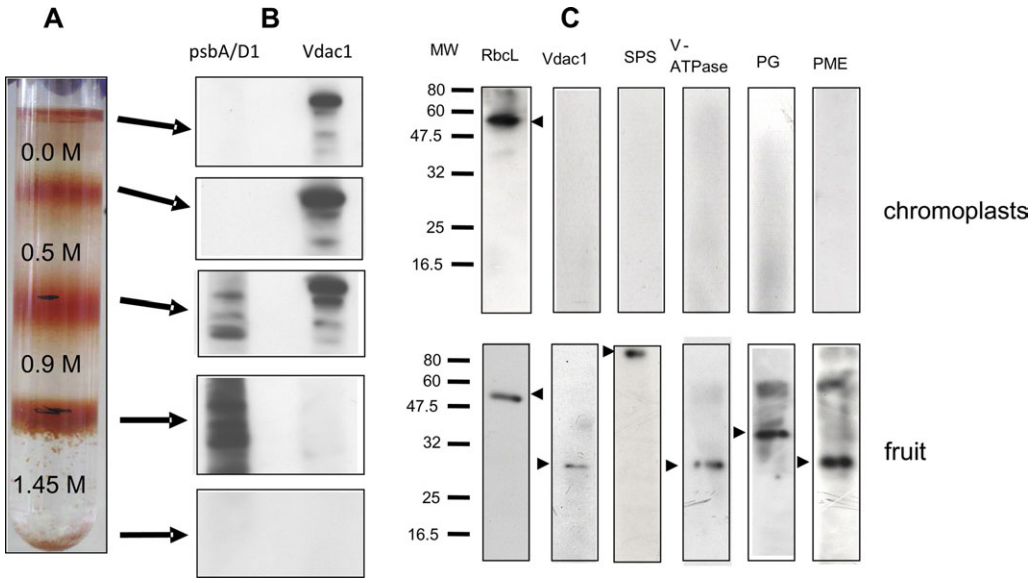

Fig. 1. Isolation, purity control, and fractionation of tomato fruit chromoplasts. (A) Separation of chromoplasts on a discontinuous sucrose gradient (0.5, 0.9, and 1.45 M); (B) Western blots for assessment of the purity of fractions at different interfaces of the sucrose gradient using antibodies for the plastidial PsBa/D1 and mitochondrial Vdac1 maker proteins; (C) Western blots for assessment of the purity of chromoplasts as compared to whole fruit proteins using antibodies against plastidial large Rubisco subunit (RbcL), mitochondrial voltage-dependent amino-selective channel protein 1 (Vdac1), cytosolic sucrose phosphate synthase (SPS), vacuolar ATPase (V-ATPase), cell wall polygalacturonase (PG) and pectin methyl esterase (PME). Arrows indicate the actual molecular weight of the marker proteins.

of wheat amyloplasts (289), rice etioplasts (240), tobacco proplastids (168), and pepper chromoplasts (151). The size of the AT plastid proteome has been estimated as approximately 2700 proteins (Millar *et al.*, 2006), indicating that we are still far from covering all the chromoplastic proteins. Despite the heterogeneity in the total number of proteins identified in each proteome, it appears (Fig. 3) that 192 (66%) proteins of the wheat amyloplast, 577 (45%) of the *Arabidopsis* chloroplast, 160 (66%) of the rice etioplast, 110 (65%) of the tobacco proplastid, and 108 (71%) of the pepper chromoplast were also present in the tomato chromoplast. Classification of the identified proteins according to MapMan allows an overview of the abundance of proteins in the various functional classes (Fig. 4). Apart from non-assigned proteins, the functions corresponding to the highest number of proteins are, by decreasing order of importance, protein-related processes, photosynthesis, amino acid metabolism, and lipid metabolism.

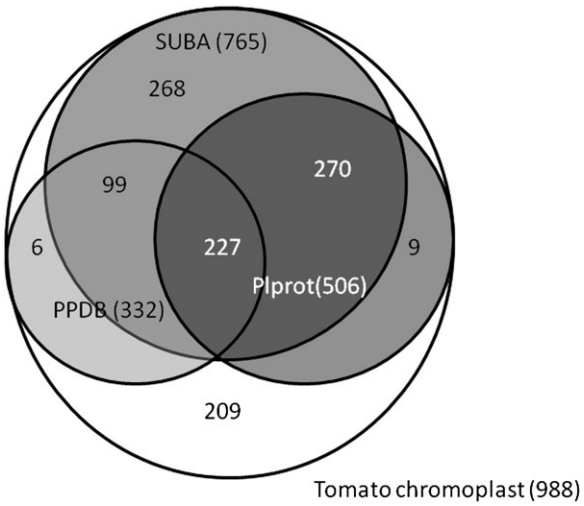

Fig. 2. Venn diagram showing the presence of tomato chromoplastic proteins in plastidial databases: SUBA, PPDB, and Plprot. Note that 209 proteins of the tomato chromoplast proteome were not listed in the databases. Comparison has been made on the basis of *Arabidopsis* annotations taking into account all tomato unigenes.

Proteins encoded by the plastid genome

The tomato chloroplastic genome comprises 84 conserved open reading frames (Kahlau *et al.*, 2006). Amongst the 84 proteins, 22 have been found in our chromoplastic proteome (Table 1), including three proteins of Photosystem II, one of photosystem I, two cytochrome B6/f proteins, four ATP synthases, one protein of the Calvin cycle (Rubisco), eight ribosomal proteins, one protein involved in protein degradation, and one acetyl CoA carboxylase. The Ycf2 protein, which corresponds to the largest chloroplast genome-encoded protein, was also identified. The *Ycf2* gene is highly expressed in chromoplasts during ripening (Richards *et al.*, 1991). Its function is not related to photosynthesis and is currently unknown. It has been shown that the Ycf2 protein plays a vital role in the plant cell (Drescher *et al.*, 2000). No RNA polymerase was detected, probably because of its low abundance.

Photosynthesis and Calvin cycle

A number of proteins involved in the PSI and PSII photosystems, in light reactions and in photorespiration were detected (Table 2), corresponding to 22% and 39% of the PSI and PSII proteins of the *Arabidopsis* chloroplast, respectively. Notably, the psbA/D1 protein, part of the core of photosystem II, has been shown to undergo rapid light-dependent degradation in chloroplasts (Mattoo *et al.*, 1984; Edelman and Mattoo, 2008). The small plastid-encoded, and the large nuclear-encoded Rubisco were also present. This is not surprising since they have also been found in

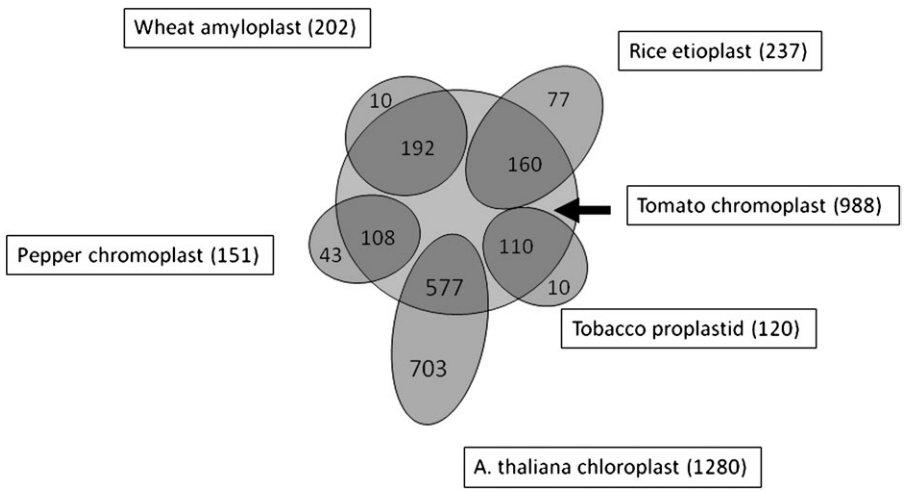

Fig. 3. Diagram showing a comparison of tomato chromoplastic proteome with other plastidial proteomes. Data from von Zyklinski *et al.* (2005) for rice etioplasts, Baginski *et al.* (2004) for tobacco proplastids, Siddique *et al.* (2006) for pepper chromoplasts, Zybailov *et al.* (2008) for *Arabidopsis* chloroplasts, and Balmer *et al.* (2006) for wheat amyloplasts. Comparison has been made on the basis of unique *Arabidopsis* annotations taking into account all unigenes.

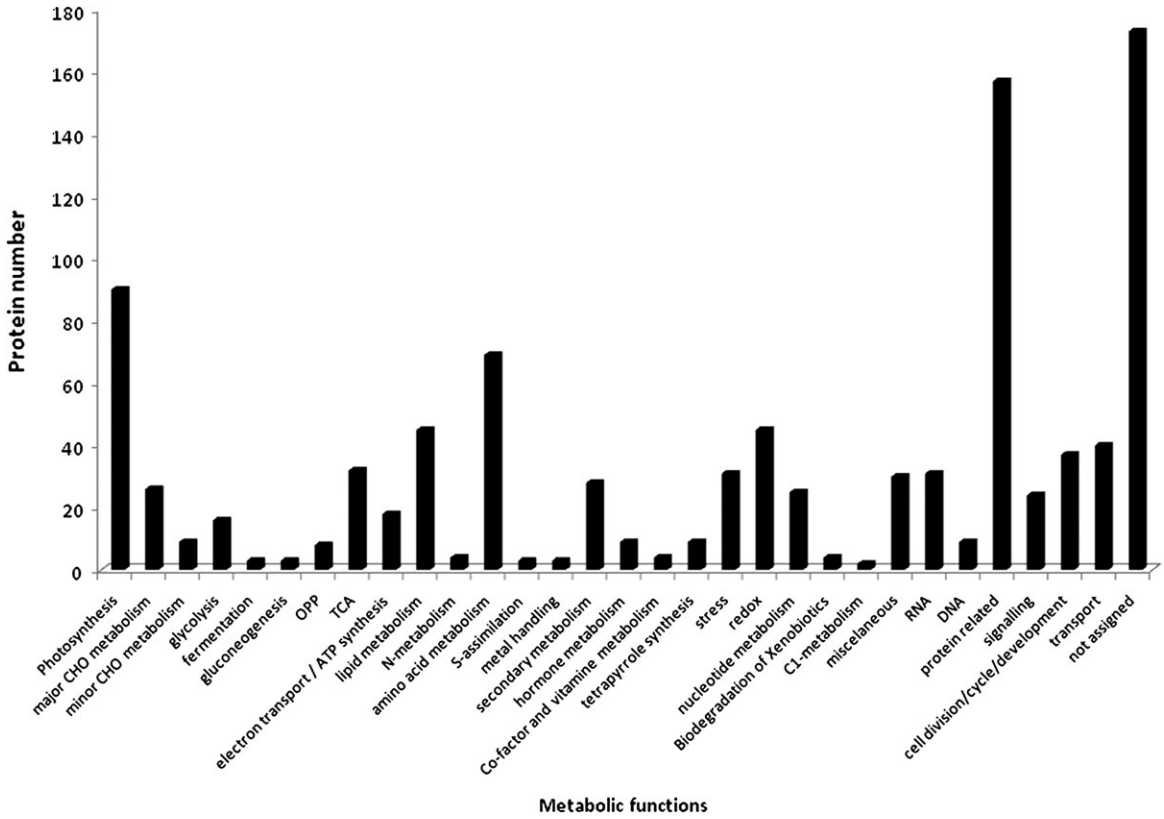

Fig. 4. Functional comparison of tomato chromoplast proteins. Proteins were assorted to their putative function by using the MapMan software (Thimm *et al.*, 2004; http://mapman.mpimp-golm.mpg.de/).

Table 1. List of proteins encoded by the tomato plastid genome encountered in the tomato chromoplast proteome
Classification has been made according to MapMan.

Plastid genome encoded proteins	GI	Plastid genome encoded proteins	GI
PSII		**Protein synthesis ribosomal protein**	
Chlorophyll binding protein psbA/D1	gi|89280615	Ribosomal protein S11	gi|89280668
Photosystem II 44 kDa protein	gi|89280631	Ribosomal protein S1	6gi|89280617,gi|89280620
Photosystem II 47 kDa protein	gi|89280661	Ribosomal protein S3	gi|89280673
PSI		Ribosomal protein S4	gi|89280637
Photosystem I P700 apoprotein A2	gi|89280634	Ribosomal protein S8	gi|89280670
PS.lightreaction.cytochrome b6/f		Ribosomal protein L16	gi|89280672
Cytochrome f	gi|89280648	Ribosomal protein L22	gi|89280674
Cytochrome b6	gi|89280665	**Protein.degradation**	
PS.lightreaction.ATP synthase		ATP-dependent Clp protease	gi|89280660
ATP synt CF0 β subunit	gi|89280621,gi|8928062	**Protein assembly and cofactor ligation**	
ATP synthase CF1 ε subunit	gi|89280641	Ycf2	gi|89280678
ATP synthase CF1 β chain	gi|89280642	**Lipid biosynthesis**	
PS.calvin cyle		Acetyl-CoA carboxylase βsubunit ACCD	gi|89280644
Rubisco large subunit	gi|89280643		

non-photosynthetic wheat rice amyloplasts (Balmer *et al.*, 2006) and rice etioplast proteomes (von Zychlinski *et al.*, 2005). The persistence of photosynthetic proteins and active Rubisco has already been reported for late stages of tomato ripening (Bravdo *et al.*, 1977; Piechulla *et al.*, 1987). In addition, a 32 kDa 'Qb binding' protein, a plastocyanin (Piechulla *et al.*, 1987), the 68 kDa subunit of PSI complex Cytf, and a CF1ATPase subunit (Livne and Gepstein, 1988) have been detected by Western blot analysis in ripe tomato fruit. The homologues of nine of the photosynthetic apparatus proteins have been detected in the plastoglobules of *Arabidopsis* and one in the plastoglobules of pepper chromoplasts (Ytterberg *et al.*, 2006). Since plastoglobules cannot be considered as a site for photosynthesis, it is

Table 2. Selected proteins identified in the tomato chromoplast proteome and discussed in the text
Classification has been made essentially according to MapMan with minor adjustments as mentioned in the Materials and methods.

Name and functional information	SGN code
1-PHOTOSYNTHESIS AND CALVIN	
Photosystem (PS)II	
Chlorophyll A-B binding prot1	U313211, U313204, U313212 , U313213, U313214
Light-harvesting Chl *a/b-binding (*LHCB6)	U312339
LHCB2.1	U312436, U312438
LHCB5	U312449
LHCB4	U312661
LHCB3	U314750
Thylakoid lumenal 29.8 kDa protein	U324760, U318255
PSII family protein	U319106
O_2-evolving enhancer protein2-1	U312571, U312572
Non-photochemical quenching4	U312967
Oxygen-evolving complex	U325341
PSII subunit O-2	U312871, U312532, U312531
PSII reaction centre PsbP fam prot	U323580,U317040
CP43 subunit of the PSII	U343039
PSI	
LHCA3	U312843
LHCA4	U312593, U317042
PSI subunit D-2	U312640
PSI subunit F	U314260
PSI subunit E-2	U313447
Cytochrome B6/f	
Photosynthetic electron transfer C	U312858
Cytochrome f apoprotein	U342407
Cytochrome b(6) subunit	U331670
Lightreactions(LR) .ATP synthase	
ATP synthase γ chain 1	U313245
ATP synthase δ chain	U313693
ATP synthase family	U313789
ATPase α subunit	U329173, U323749
ATPase F subunit	U340510
LR. electron carrier (ox/red)	
DNA-damage-repair/toleration protein	U312690
NADPH dehydrogenase/oxidoreductase	U314955
Autophagy	
Senescence-associated gene(SEN1)	U316277
Tetrapyrolle synthesis	
Glutamyl-tRNA(Gln) amidotransferase	U323134
Ferrochelatase I	U316403
Glutamate-1-semialdeh 2,1-aminomutase	U315915
Sirohydrochlorin ferrochelatase	U323001
Porphobilinogen synthase	U312668
Hydroxymethylbilane synthase	U315567
Uroporphyrinogen decarboxylase	U315267
Coproporphyrinogen oxidase	U315993
Protoporphyrinogen oxidase	U326999
Chlorophyll breakdown	
Pheophytinase	U317890
phaeophorbide a oxygenase	U313134
Stay-green protein 1	U316068

Table 2. *Continued*

Name and functional information	SGN code
Calvin cycle: RuBisCO and RuBisCO related	
Large subunit of Rubisco	gi|89280643,U344009, U346314
Rubisco small subunit 1A	U314254, U314262
Rubisco small subunit 3B	U314700, U314701, U314722, U338973
Rubisco activase	U312543, U312544
Rubisco large subunit N-methyltransferase	U326460
Chaperonin 60α	U312538, U312542
Chaperonin 60β	U316742
Calvin cycle: others	
Ribulose-PH 3-epimerase	U313308
Phosphoribulokinase	U312791
Phosphoglycerate kinase	U313176
Glycerald-3-PH DH β	U312802, U312461, U312804
Triose-PH isomerase	U313729
Fructose-bisPH aldolase	U314787, U314788, U312344, U312608, U312609
Fructose-1,6-bisphosphatase	U316424
OPP pathway	
Ribose 5-phosphate isomerase	U315528
Transketolase, putative	U312320, U312319, U312322, U323721
Transaldolase	U315742, U315064
2. CARBOHYDRATE METABOLISM	
Glucose 6-phosphate transporter	U330538
Triose phosphate translocator	U312460
Starch synthase I	U318293
ADP-glucose pyrophosphorylase	U317866
1,4-α-glucan branching enzyme	U312423, U312427
α-amylase3	U317456 ; U326232, U326817
β-amylase 3	U313315
Glucan phosphorylase	U316416, U316417, U325849, U345057
Phosphoglucan, water dikinase	U328612
Disproportionating enz 1	U322816, U333138, U342143
Disproportionating enz 2	U327405
Isoamylase3	U328875, U333011
Starch excess 1	U315116
Starch excess 4	U317732
3. LIPID SYNTHESIS AND METABOLISM	
Fatty acid synthesis	
Pyruvate dehydrogenaseE1α	U313753
Pyruvate dehydrogenaseE1β	U314162
Pyruvate dehydrogenaseE2	U317019
Biotin carboxyl-carrier prot CAC1	U317261, U317459
Biotin carboxylase subunit 2 CAC2	U324109, U327019
Acetyl-CoA carboxylase CAC3	U323169, U317741
Acetyl-CoA carboxylase βsubunit ACCD	gi|89280644
S-malonyltransferase	U316102
3-Ketoacyl-acyl carrier prot synth I	U315474, U315475, U325875
3-Ketoacyl-acyl carrier prot synth III	U316868, U335865
3-Oxoacyl-ACPreductase	U315110

Table 2. *Continued*

Name and functional information	SGN code
β-Hydroxyacyl-ACP dehydratase	U319205
enoyl-ACP reductase	U321872,U315697
Phospholipid synthesis	
Trigalactosyldiacylglycerol2	U321141
Plastid transcriptionally active4=VIPP1	U317521
Lipid catabolism	
Phospholipase D α 1	U316492, U318835
LOXC	U315633
Hydroperoxide lyase	U315884
Alcohol dehydrogenase2	U314358
4. AMINO ACID METABOLISM	
Shikimate pathway	
3-Deoxy-7-phosphoheptulonate synthase	U319272
Shikimate 5 DH	U318401, U321940
3-Phosphoshikimate 1-carboxyvinyltransf.	U317466
Chorismate synthase	U313391
Aromatic aa.tryptophan synthesis	
Anthranilate synthase β subunit. 1	U330695
Anthranilate synthase α2	U321504, U321505
Anthranilate phosphoribosyltransf	U321976
Tryptophan indole-3-glycerol PH synthase	U318203
Tryptophan synthase, α subunit	U317564
Tryptophan synthase, β subunit	U317245
Methionine synthesis	
Homoserine kinase	U321893
Threonine synthase	U316421,330577
Aspartate semialdehyde DH	U320340
Aspartate kinase/ homoserine DH	U317344
Cystathionine β-lyase	U320667
Sulphur assimilation	
Sulphite oxidase	U314329
ATP sulphurylase 3	U313496, U313497
Ammonia assimilation	
Glutamine synthetase	U314517
Ferredoxin dep glutamate synth 1	U317540, U323261
nitrate reductase	U317524
5. TERPENOID METABOLISM	
Rohmer (non-mevalonate) pathway	
1-Deoxyxylulose 5-PH synthase	U316258
Photorespiration	
Phosphoglycolate phosphatase	U314994
(*S*)-2-hydroxy-acid oxidase	U312724, U333873
Glycine cleavage system H	U312985
Serine hydroxymethyl transferase3	U319359, U319360
Mevalonate pathway	
Acetoacetyl-Coa thiolase 2	U314024
Isopentenyl-diPH delta-isomerase	U315069
Prenyl transferases	
Geranylgeranyl pyroPH s synthase	U326688, U325914
Geranylgeranyl reductase	U316915,U313450
Lycopene biosynthesis	
Phytoene synthase 1	U314429
Phytoene desaturase	U318137
Zeta-carotene desaturase	U335523,U316184
6. VITAMIN BIOSYNTHESIS	
Riboflavin (vitamin B2)	
GTP cyclohydrolase II	U317027

Table 2. *Continued*

Name and functional information	SGN code
6,7-dimethyl-8-ribityllumazine synthase	U322093
Lumazine-binding family protein	U323258
Panthothenate (vitamin B5)	
Beta-ureidopropionase	U321324
Folate (vitamin B9)	
4-Amino-4-desoxychorismate lyase	U314873
Folate metabolism	
Serine hydroxymethyltransferase	U315084
Tocopherols (vitamin E)	
Methyltransferase	U313381
γ-tocopherol methyltransferase activity	U317964
7. REDOX	
Ascorbate-glutathione cycle	
Stromal L-ascorbate peroxidase	U314092
Thylakoid L-ascorbate peroxidase	U314093
Glucose-6-PH dehydrogenase2	U317444
Glucosamine/galactosamine-6-PH isomerase	U315096, U315098, U318386
Phosphogluconate dehydrogenase	U316131
6-phosphogluconate DH	U318328, U332994
Glutathione peroxidase	U315143, U315728
Monodehydroascorbate reductase	U320487, U345138
Dehydroascorbate reductase	U313719, U313537
Peroxiredoxin Q	U314061
peroxiredoxin type 2	U314448
2-cys peroxiredoxin	U314924,U314923
Thioredoxin M-type 1	U329463
Thioredoxin M-type 4	U316173,U318067
Thioredoxin oprotein2	U319145
Thioredoxin reductase	U324098
Redox enzymes	
Catalase	U323590, U312411, U323759
Cu, Zn superoxide dismutase 2	U315383, U315384
Cu/Zn superoxide dismutase 1	U317104
Fe-superoxide dismutase	U313819, U317645, U319423, U314438
NADH-ubiquinone o/r 24 kDa	U316255
NADH-ubiquinone o/r 51 kDa	U316563
NADH-ubiquinone o/r 20 kDa	U343941
NADH-ubiquinone o/r 75 kDa	U315458
8-HORMONES	
IAA synthesis	
Tryptophan indole-3-glycerol PH synthase	U318203
Anthranilate synthaseβ subunit. 1	U330695
Anthranilate synthase α2	U321504, U321505
ABA synthesis	
Zeaxanthin epoxidase	U321035
Phosphatidylglycerolphosphate synthase	U319207
Glycolipid and sulpholipid synthesis	
UDPsulphoquinovose synthase	U317216, U317217
UDP-glucose:sterol glucosyltransferase	U317386, U330998
Short-chain dehydrogenase	U326257
ABA catabolism	
(+) Abscisic 8'hydroxylase	U325016
Jasmonates synthesis	
LOX2	U315633

Table 2. *Continued*

Name and functional information	SGN code
LOX3	U321151
Allene oxide synthase	U319339
Allene oxide cyclase	U316550
9. SIGNALLING ELEMENTS	
Glucose signalling	
Hexokinase1	U328823, U335794
Calcium signalling	
Calmodulin-binding heat-shock	U327931
IQ calmodulin-binding	U324954
Calnexin 1	U315861
Ca-binding EF hand fam	U318939
10. STRUCTURAL AND BUILDING BLOCKS	
Plastid lipid associated	
PAP / fibrillin fam protein	U341818, U319404, U321757,U319105, U316479, U316793, U313480
FTSZ1-1	U320588, U328370
FTSZ2-2	U324505
11- PROTEIN IMPORT SYSTEM	
Toc/Tic	
Tic32-IVb	U314839
TIC110	U345264, U325822
Toc75-III	U316483
Toc 75 V	U316769
1-Deoxy-d-xylulose 5-PH reductoisomerase	U315947
2C-MethylDerythritol 2,4cyclodiPH synt	U318786
2C-MethylDerythritol PH cytidyltransferase	U319197
4-Hydroxy3methylbut2en1yl diPH synt	U314139
Tic62	U318707,U317146
Tic55	U313134
Tic40	U328395
Tic20 II	U316271
Chaperonin associated machinery	
Heat shock protein 70-1	U313975
Heat shock protein 70-7	U315717
Heat shock protein 93-V	U312782, U312783, U312784
60 kDa chaperonin α subunit	U312538, U312542
60 kDa chaperonin β subunit	U316742
Signal peptide peptidase (SPPA)	U341182
Proteins translocated to the lumen	
LHCP3	U314750
LHCP5	U312449
LHCP6=CP24	U312339
23 kDa protein	U312571
Rieske protein	U313134
PC=DNA-damage-repair/toleration prot 112	U312690
PSI subunit F	U314260
Cfo-II= chloroplastic quinone-o/r	U313789
CeQORH	U314190
12. VESICULAR TRANSPORT	
COPII	U318911
Clathrin heavy chain	U321296, U346835
Coatomer β subunit	U315366
Coatomer γ2 subunit	U327299

probable that either full size or segments of non-functional proteins are stored in the plastoglobules after the disintegration of photosynthesis complexes in the chromoplast. The absence of a large number of proteins involved in PSI and PSII could be related to an autophagy process similar to that described for senescent leaves. Interestingly, the SEN1 gene, described as being involved in autophagy (Wada *et al.*, 2009) has also been found here.

Chemical analysis performed on fruit at the breaker+10 stage of ripening indicated undetectable levels of chlorophyll *a* and *b*. Consistent with the absence of chlorophyll in the chromoplasts, all steps of the chlorophyll biosynthesis branch were lacking (see Supplementary Fig. S1 at *JXB* online), including the magnesium-chelatase, which is at the cross-roads of the branch. This is consistent with the absence of photosynthetic activity in fruit at this stage of development (Piechulla *et al.*, 1987). Only proteins leading to the synthesis of protoporphyrin IX were found, with the exception of glutamyl tRNA reductase. Among the three proteins of the haem-derived pathway leading to phytochromobilin, involved in phytochrome synthesis and described as plastid-localized (Terry and Lagarias, 1991), only the protein of the first step was recovered in the chromoplast proteome. Concerning the protoporphyrin pathway, one protein out of three of the sirohaem branch was detected. This branch provides the cofactor for sulphite reductase, involved in the assimilation of sulphur and its incorporation into sulphur amino acids, as well as for nitrite reductase, involved in the assimilation of nitrogen.

Interestingly, the chromoplast proteome comprises several proteins known to participate in chlorophyll catabolism. These include proteins directly involved in the breakdown of chlorophyll, pheophytinase (Schelbert *et al.*, 2009) and pheophorbide a oxygenase (Pruzinska *et al.*, 2005) and a regulator of pheophorbide a oxygenase, the stay-green protein, sgr1 (Ren *et al.* 2007). The presence of these proteins in fully-developped chromoplasts, assuming they are enzymatically active, indicates that the chromoplast could comprise chlorophyll breakDaown processes similar to those occuring during the senescence leaf chloroplasts (Thomas *et al.*, 2009).

Most of the proteins of the Calvin cycle were identified in the tomato chromoplast including Rubisco. Four different proteins of the small nuclear-encoded subunits of Rubisco, probably encoded by four different genes (proteins annotated as 3B subunits are in fact different) and three fragments of the plastid-encoded large subunit have been found. Also, a Rubisco activase, a Rubisco large subunit N-methyltransferase and two chaperonins (60α and 60β) were detected. This means that all components necessary for Rubisco activity are present. Almost all proteins of the OxPPP pathway are represented in the chromoplast proteome (Table 2), consistent with the presence of active OxPPP in ripening fruit and in pepper fruit chromoplasts (Thom *et al.*, 1998). Although proteome analysis alone cannot provide evidence of the functionality of the Calvin cycle, the persistence of all the proteins of this pathway suggests a possible role in metabolic adjustments that would

provide not only reductants but also precursors of nucleotides (from ribose-5-phosphate) and aromatic amino acids (from erythrose-4-phosphate) to allow the OxPPP cycle to function optimally. Alternatively, the presence of the Calvin cycle part of the photosynthesis machinery may simply represent a left-over corresponding to the recovery of the photosynthetic activity required for converting the chromoplast back to the chloroplast. This has already been observed in many plant tissues, including fruit (Hudák *et al.*, 2005).

Carbohydrate metabolism

Sugars derived from photosynthesis within the fruit are extremely limited at the approach of fruit maturity. The bulk of sugar accumulation comes mainly from transport through the phloem. The chromoplast has the potential to translocate sugars via a membrane located glucose 6-phosphate transporter and triose phosphate/phosphoenol pyruvate translocator. Due to the absence of photosynthetic activity the reducing power of the chromoplast may be satisfied by the light-independent production of NADPH through the glucose-6-phosphate dehydrogenase (G6PDH) and 6-phospho gluconate dehydrogenase (6PGDH) proteins of the oxidative pentose phosphate pathway, OxPPP (Kruger and van Schaewen, 2003). Previous biochemical data have characterized functional OxPPP and import of G6P in isolated sweet pepper (Thom *et al.*, 1998) and buttercup (Tetlow *et al.*, 2003) chromoplasts. In addition to proteins of the OxPPP, it was mentioned earlier that the Rubisco protein and all proteins of the Calvin cycle have been found in tomato chromoplasts. If all these pathways are active, this could suggest that CO_2 generated by the OxPPP could be re-incorporated metabolically. Although such a possibility remains to be demonstrated in the chromoplast by enzymatic and metabolic analysis, reassimilation of CO_2 generated from OxPPP by Rubisco has clearly been shown in non-photosynthetic oil-accumulating seeds in order to sustain fatty acid biosynthesis (Schwender *et al.*, 2004).

Proteins of the starch biosynthesis pathway were also identified including soluble starch synthase, ADP-glucose pyrophosphorylase, and 1,4-alpha-glucan branching protein. Starch grains have been observed in flower chromoplasts, especially in tissues grown *in vitro* (Keresztes and Schroth, 1979) indicating the functionality of the biosynthetic system. However, several proteins involved in starch degradation were also found such as α-amylase 3, β-amylase 3, glucan phosphorylase, phosphoglucan, water dikinase, disproportionating enzymes 1 and 2, and isoamylase 3, thus suggesting a rapid turnover of starch. In addition, starch excess proteins 1 and 4 (sex1 and 4) regulating starch accumulation were also present. *Arabidopsis* mutants for sex proteins accumulate an excess of starch (Yu *et al.*, 2001). Interestingly, neither starch-degrading protein nor starch excess protein have been reported in the proteome of wheat amyloplasts where a high accumulation of starch occurs (Balmer *et al.*, 2006).

Lipid synthesis and metabolism

Chromoplasts possess the entire metabolic equipment for the synthesis of 3-oxoacyl-ACP, the precursor of fatty acids (Table 2; see Supplementary Fig. S2 at *JXB* online). Interestingly, almost all the subunits of acetyl CoA carboxylase were detected (three different proteins of the nuclear-encoded subunits corresponding to four different genes, CAC1, CAC2, and CAC3 and one plastid-encoded subunit, ACCD). Key proteins for the synthesis of phospholipids, glycolipids, sulpholipids, and sterols were also identified (Table 2). If all these proteins are enzymatically active, these results indicate that the chromoplast has the ability to synthesize fatty acids and polar lipids such as sulpholipid and phosphatidylglycerol, probably in co-ordination with the endoplasmic reticulum (Andersson *et al.*, 2007). The presence of the trigalactosyldiacylglycerol 2 protein, a permease-like component of an ABC-transporter involved in ER-to-thylakoid trafficking (Awai *et al.*, 2006) reinforces this hypothesis. A protein involved in vesicular transport from the inner envelope to thylakoids, 'plastid transcriptionally active 4' (VIPP1) was also present (Kroll *et al.*, 2001), which is consistent with the presence of intense vesicular activity during chromoplast formation as shown by electron microscopy (Westphal *et al.*, 2001).

Many proteins involved in lipid metabolism were recovered in the chromoplast proteome (corresponding to category 11.9 in Supplementary Table S1 at *JXB* online). Of special interest is the presence of all proteins potentially involved in the LOX pathway, leading to the generation of aroma volatiles, including phospholipase D α 1, lipoxygenase C (Chen *et al.*, 2004,), and hydroperoxide lyase. In addition, an alcohol dehydrogenase 2 capable of interconverting aldehydes and alcohols is present. This protein has been shown to participate in aroma formation in tomato (Speirs *et al.*, 1998). It is therefore possible to assign a role of the chromoplast in the synthesis of LOX-derived volatiles which are known to be synthesized at a high level in ripe red fruit (Birtic *et al.*, 2009) at a stage where LOX-C gene expression is still high (Griffiths *et al.*, 1999).

Proteins related to transcription, translation, and post-transcriptional modifications

This category comprises 121 proteins that have not been reported in Table 1 but are listed in Supplementary Table S1 at *JXB* online under the categories 27.1 to 29.2.5 according to MapMan. These proteins, potentially involved in transcription, translation, folding, assembly, turnover, and protein storage, represent the major functional group found in chromoplasts (Fig. 4). No RNA polymerase has been detected among the sequenced proteins encoded either by the nucleus or by the chromoplastic genome. This indicates that the transcriptional activity at this stage of ripening was probably very low. This is consistent with the progressive decline in the overall rate of RNA synthesis observed throughout chromoplast development in ripening tomato fruits (Marano and

Carillo, 1992). The absence of detectable RNAse exonuclease II which is thought to participate in the RNA degradation pathway could account for higher stability of the RNA. Sustained transcriptional activity has been measured in chloroplasts (Briat *et al.*, 1982), but a 5–10-fold decrease in activity for most plastid genes was observed in chromoplasts (Deng and Gruissem, 1987). Other experiments did not report such major variations in the relative transcription rate (Marano and Carrillo, 1992; Kahlau and Bock, 2008), except for the up-regulation of the *trnA* gene (encoding the tRNA-Ala) and the *rpoC2* gene (encoding an RNA polymerase subunit) and a significant up-regulation of the *acetyl-CoA carboxylase* gene (*ACCD*), the only plastid-encoded gene involved in fatty acid biosynthesis (Kahlau and Bock, 2008). The plastid-encoded ACCD was found in the tomato chromoplast proteome analysed in this study. Seventeen transcription factors were detected (category 27.3 in Supplementary Table S1 at *JXB* online), nine of these having a plastid signal. This low number of these factors is probably related to the low number of chromoplastic genes requiring regulation. However, they may play an important role in signalling of the nucleus to the plastids (anterograde signalling). Plastids have 70S ribosomes comprising 50S and 30S subunits (Yamaguchi and Subramanian, 2003). These ribosomal proteins are represented by seven nuclear-encoded proteins of the 50S fraction and nine of the 30S fraction (29.2.1.1 category in Supplementary Table S1 at *JXB* online), indicating that the translational machinery is present. However, our data cannot tell whether this machinery is functional. The presence of 13 tRNA synthases or ligases (29.1 category), eight elongation factors (29.2.4 category), and numerous chaperonins are additional indications of translational activity. As already found for bell pepper (Siddique *et al.*, 2006), tomato chromoplasts contain a translation inhibitor protein of the L-PSP type (SGN-U317502) presumed to have endonuclease activity towards mRNAs that might prevent the translation of certain proteins that are no longer required in chromoplast function. Most plastid genes are transcriptionally down-regulated during chromoplast development, especially photosynthesis-related genes. The *ACCD* gene, which is involved in fatty acid biosynthesis, is the only plastid-encoded gene showing stable expression in chromoplasts (Kahlau and Bock, 2008). Interestingly, functional ribosomes and translation activity have been observed in tobacco plants in which the plastid RNA polymerase genes have been disrupted (De Santis-Maciossek *et al.*, 1999). Therefore, the undetectable levels of RNA polymerase in tomato chromoplast seems compatible with the presence of active translational activity.

Amino acid metabolism

Four of the six proteins of the shikimate pathway (Herrmann and Weaver, 1999) have been identified: 3-deoxy-7-phosphoheptulonate synthase, shikimate 5-dehydrogenase, 3-phosphoshikimate 1-carboxyvinyltransferase, and chorismate synthase. The final step of the pathway produces chorismate, the precursor of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. The presence of this pathway within the chloroplast has already been suggested by Herrmann and Weaver (1999). Confirmation of the synthesis of the three amino acids in the chromoplast is provided by the fact that many of the proteins involved in the aromatic amino acid biosynthetic pathway are present in the tomato chromoplastic proteome, especially those of the final step: anthranilate synthase, anthranilate phosphorybosyltransferase, tryptophan indole-3-glycerol phosphate synthase, tryptophan synthase α and β subunit. The synthesis of methionine is known to be linked to the aspartate pathway and to the assimilation of sulphur and incorporation in cysteine (Hesse and Hoefgen, 2003). In the tomato chromoplastic proteome almost all of the proteins of this pathway have been encountered: homoserine kinase, threonine synthase, aspartate semialdehyde dehydrogenase, and bi-functionnal aspartate kinase/homoserine dehydrogenase. The intracellular localization of the final step of methionine synthesis is a matter of debate (Hesse and Hoefgen, 2003; Ravanel *et al.*, 2004). Among the chromoplastic proteins, cystathionine beta-lyase has been identified, a clear indicator of the synthesis of methionine within the chromoplast. One *Arabidopsis* isoform was also found to be located in the chloroplast (Ravanel *et al.*, 2004). The presence of proteins involved in the early steps of sulphur assimilation and in the assimilation of ammonia in tomato chromoplasts is indicative of the capability to assimilate sulphur and ammonia.

Terpenoid metabolism

The large majority of the proteins of the Rohmer pathway (non-mevalonate or methylerythritol phosphate pathway) leading to the synthesis of terpenoid precursors in plastids are present in the tomato chromoplast (Table 2) except for the 4-diphosphocytidyl-methylerythritol kinase. The mevalonate pathway is represented by one protein, acetoacetyl-CoA thiolase 2. Both the Rohmer and the mevalonate pathways lead to the formation of the C5 compounds isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate which can be interconverted by IPP isomerase, also present in the tomato chromoplastic proteome. The following steps involve prenyl transferases. Two geranylgeranyl pyrophosphate (GGPP) synthase (synthesis of the C20 precusor of carotenoid, gibberellins and side chains of tocopherols, phytol, and phylloquinone) and two geranylgeranyl reductases have also been identified. They could participate in the reduction of geranylgeranyl-chlorophyll to chlorophyll *a* and also of free geranylgeranyl diphosphate into phytyl diphosphate, which is used for chlorophyll, tocopherol, and phylloquinone synthesis (Zybailov *et al.*, 2009).

Chemical analysis performed on fruit at the breaker+10 stage of ripening showed the prevalence of lycopene (all-*trans* and, as a minor form, a *cis* isomer). Beta-carotene, a compound tentatively identified as γ-carotene and lutein

were also detected (in decreasing order of content), as well as traces of other compounds (data not shown). Almost all proteins dedicated to the biosynthesis of lycopene have been identified among the chromoplastic proteins: phytoene synthase 1, phytoene desaturase, and two zeta-carotene desaturases. Interestingly tomato has two phytoene synthases, a chloroplastic PSY-2 which is expressed in green tissues and green fruit and a chromoplastic PSY-1 which strongly accumulates during fruit ripening (Fraser *et al.*, 1999). The presence of only PSY-1 in our set of proteins is therefore consistent with the metabolic data previously published. Surprisingly, no sequence for the plastid terminal oxidase (PTOX), a co-factor for carotene desaturases, has been identified, although data indicate that a mutant deficient in this protein is severely impaired in lycopene synthesis during tomato fruit ripening (Shahbazi *et al.*, 2007). The most likely explanation is that PTOX is present at such a low abundance that it has not been detected in our proteomic analysis. Downstream proteins, namely lycopene cyclases (lycopene β-cyclase, lycopene ϵ-cyclase, and carotenoid hydroxylases (β-carotene hydroxylase and carotenoid ϵ-hydroxylase) were not identified and are therefore either totally absent or present at undetectable levels. This is in agreement with data showing that the accumulation of lycopene is due to a down-regulation of the genes encoding downstream proteins of this pathway (Ronen *et al.*, 1999) thus leading to a weak metabolic flux towards the synthesis of beta carotene and xanthophylls. However, a zeaxanthin epoxidase, catalysing the synthesis of violaxanthin was found, which is consistent with the presence of violaxanthin in tomato chromoplasts.

Biosynthesis of vitamins

It is known that the chloroplast is the site for the synthesis of thiamine, vitamin B1 (Julliard and Douce, 1991). However, none of the proteins involved in thiamine biosynthesis were detected in the tomato chromoplast proteome. Several proteins of riboflavin (vitamin B2) biosynthesis (Roje, 2007) have been identified in the tomato chromoplastic proteome: GTP cyclohydrolase II, 6,7-dimethyl-8-ribityllumazine synthase, and lumazine-binding family protein similar to riboflavin synthase. These data confirm the predictions made so far by both experimental and bio-informatic analysis (Roje, 2007). Only one protein of the folate (vitamin B9) biosynthesis pathway has been encountered. The active protein catalyses the conversion of chorismate to para-aminobenzoate: 4-amino-4-desoxychorismate lyase and is part of the pathway already known to be located in the plastids (Bedhomme *et al.*, 2005).

The early steps of the biosynthesis of the side chain of tocopherols (vitamin E), which are in common with other metabolic pathways (terpenes, sterols, carotenoids…), are present in the chromoplasts and discussed in the lipid section. Regarding the other branches of the pathway, our data show the presence of the methyltransferase converting methyl phytylquinol to the gamma/alpha-tocopherol branch (at the expense of the delta/beta-tocopherol branch) and the final protein of this branch, gamma-tocopherol methyltransferase activity, involved in conversion of gamma tocopherol to α-tocopherol formation. The latter protein has already been described in the *Capsicum* chromoplast (d'Harlingue and Camara, 1985). The apparently greater abundance of these two proteins from the tocopherol pathway may explain the large prevalence of alpha-tocopherol and to a lesser extent gamma-tocopherol, and the virtual absence of delta and beta-tocopherol in tomato chromoplast extracts. Chemical analysis performed on fruit at the breaker +10 stage of ripening revealed the presence 2.25 μg g^{-1} FW of α-tocopherol and 0.15 μg g^{-1} of γ-tocopherol. The tocopherol cyclase, VTE1, which has been identified in chloroplast plastoglobules of *Arabidopsis* (Vidi *et al.*, 2006) has not been encountered in the tomato chromoplast proteome.

Redox proteins

Reactive oxygen species appear to regulate carotenoid synthesis in chromoplasts (Bouvier *et al.*, 1998) and redox systems are considered to have several functions in the plastids, including plastoglobule protection, pathogen defence, stress response, protection against reactive oxygen species, signalling, and energy (Foyer and Noctor, 2003). As many as 21 proteins of the ascorbate–glutathione cycle have been detected (Table 2). They include key components of the cycle such as stromal and thylakoid-bound L-ascorbate peroxidase, glutathione peroxidase, monodehydroascorbate reductase, and dehydroascorbate reductase. In addition, three types of peroxiredoxins and four types of thioredoxins have been identified. The thioredoxin reductase encountered in the chromoplast may be indicative of the presence of the so-called NADPH-dependent thioredoxin system. The number of proteins involved in redox reactions is also significant, including several catalases, superoxide dismutases, peroxidases not belonging to the class of ascorbate peroxidases, and NADH-ubiquinone oxidoreductases. The proton-pumping NADH:ubiquinone oxidoreductase, also called complex I, is the first of the respiratory complexes providing the proton motive force essential for the synthesis of ATP. The presence of a chemiosmotic ATP synthesis has been demonstrated in the chloroplast (Morstadt *et al.*, 2002) which is linked to a redox pathway and potentially involved in carotene desaturation and membrane energization. Closely related forms of this complex exist in the mitochondria of eukaryotes and in the plasma membranes of purple bacteria (Friedrich *et al.*, 1995). Such an important list of redox proteins indicates that chromoplasts have integrated antioxidant defence/protection machinery, similarly to chloroplasts (Giacomelli *et al.*, 2007) with sometimes dual targeting to the mitochondria (Chew *et al.*, 2003). In support of the presence of a functional redox system in chromoplasts, it has been demonstrated that the activity of superoxide dismutase and proteins of the ascorbate–glutathione cycle was up-regulated during ripening of pepper fruit (Marti *et al.*, 2009).

Hormones

Proteins involved in the synthesis of several hormones have been encountered. Homologues of the alpha and beta subunits of anthranylate synthase of *Arabidopsis* are present in the tomato chromoplasts. They are involved in the biosynthesis of tryptophan and have been described as key elements in the regulation of auxin production. The encoding genes are ethylene responsive, which makes a link between ethylene and auxin (Stepanova *et al.*, 2005). IAA synthesis has been proposed to occur via a cytosolic tryptophan-dependent (indole-3-acetaldoxime) and a plastidial tryptophan-independent (indole-3-glycerophosphate) pathway. In the tomato chromoplast, only an indole-3-glycerol phosphate synthase involved in the tryptophan-independent patway was encountered (Ouyang *et al.*, 2000). Two proteins of the ABA pathway have been identified: zeaxanthin epoxidase and short-chain dehydrogenase indicating that chromoplasts could be active in producing ABA. However, the absence of the 9-*cis*-epoxycarotenoid dioxygenase may be indicative of the low activity of the ABA biosynthetic pathway coincident with the decrease in ABA content well before the climacteric peak in tomato (Martinez-Madrid *et al.*, 1996). The three proteins involved in the early steps of the biosynthesis of jasmonates which are present in the chloroplast (Delker *et al.*, 2007) have also been identified in the tomato chromoplast: lipoxygenases 2 and 3, allene oxide synthase, and allene oxide cyclase. The final steps occur in the peroxisome (Delker *et al.*, 2007). Proteins involved in the formation of the gibberellin skeleton (*ent*-copalyl diphosphate synthase, and *ent*-kaurene synthase) are known to be present in the chloroplast (Railton *et al.*, 1984). They were not found among the tomato chromoplastic proteins, suggesting the absence or low level of gibberellin biosynthesis.

Signalling elements

Two hexokinase1 homologues that could potentially participate in glucose signalling are present in the tomato chromoplast. In *Arabidopsis*, hexokinase1 has been located to the mitochondria (Rolland and Sheen, 2005) but, in spinach, hexokinase activity has been found in plastids (Wiese *et al.*, 1999). Chloroplast-to-nucleus signalling (retrograde signalling) can be mediated by reactive oxygen species (ROS), Mg-protoporphyrin IX as well as by secondary messengers such as Ca^{2+} (Surpin *et al.*, 2002). Many proteins involved in ROS have been identified. Mg-Protoporphyrin IX plays an important role in retrograde signalling (Strand *et al.*, 2003) by inhibiting the expression of the nuclear genes involved in photosynthesis. However, no magnesium-chelatase has been detected here indicating that the synthesis of Mg-protoporphyrin IX is probably not very active in chromoplasts. This is consistent with the fact that down-regulation of genes involved in photosynthesis occurs at the early stages of fruit ripening. Several elements of the calcium signalling pathway have been encountered including calmodulin, calnexin, and a calcium-binding EF hand family protein (Table 2).

Structural and building blocks

Seven out of the 10 tomato fibrillin-type lipid-associated proteins expected from genomic data (Laizet *et al.*, 2004) have been identified. A number of these proteins have been found in the chloroplast thylakoid proteome, as well as in plastoglobules, where they play a structural role (Austin *et al.*, 2006; Ytterberg *et al.*, 2006). Over-expression in tomato fruit of one of these proteins originating from *Capsicum annuum*, involved in the formation of carotenoid-storing fibrils, has been shown to increase carotenoid content, but without fibril formation, and transiently to delay thylakoid disappearance in tomato fruit (Simkin *et al.*, 2007). Homologues of the three FtsZ proteins from *Arabidopsis*, which fall into two classes, FtsZ-1 and FtsZ-2, have been identified. These proteins are seen as plastid-located tubulin ancestors. They are involved in plastid division, which is unlikely to occur at this particular fruit development stage. In addition to their stromal location, FtsZ-1 is also present in thylakoids, especially in young chloroplasts, while FtsZ-2 is also found in the chloroplast envelope. Evidence exists for a functional difference between the two FtsZ classes which may not be limited to plastid division (El-Kafafi *et al.*, 2008). This may explain their presence in chromoplasts.

Protein import system

Studies on the targeting of nuclear-encoded proteins have defined several pathways to and within the chloroplast (Jarvis, 2008). For accessing the membranes or interior of chloroplast, a Toc/Tic (Translocon at the outer/inner envelope membrane of chloroplast) import machinery is present for the translocation of proteins carrying a transit peptide. Among the Toc complexes, only two of them have been found: Toc64-V and two subunits of Toc75 (Toc75-III and Toc75-V). The latter two proteins were the only Toc75 homologues to be identified at the protein level. Toc75-III is universally expressed in all plant tissues and is therefore believed to be the main import pore of the Toc complex (Vothknecht and Soll, 2005). It is totally embedded in the membrane and functions as a channel through which proteins cross the outer membrane. Toc64 is an accessory member of the Toc complex which serves as docking and guidance for facilitating access to the translocation machinery. Notably, core components such as TOC159 and Toc34, characterized as precursor protein receptors, are absent. Contrary to Toc, many of the proteins proposed to be components of the Tic system by Jarvis (2008) or predicted by Kalanon and McFadden (2008) are present in the chromoplast proteome described in this study (Fig. 5). In addition, several proteins of the chaperonin-associated machinery, including an Hsp70 group an Hsp93 and two Cpn60 proteins (Cpn60A and Cpn60B) have been identified. A signal peptide peptidase, SPP, has also been identified. Internal trafficking for transport through the thylakoid to the lumen is mediated by several mechanisms: Sec-, SRP- , Tat-dependent, and spontaneous. None of the proteins

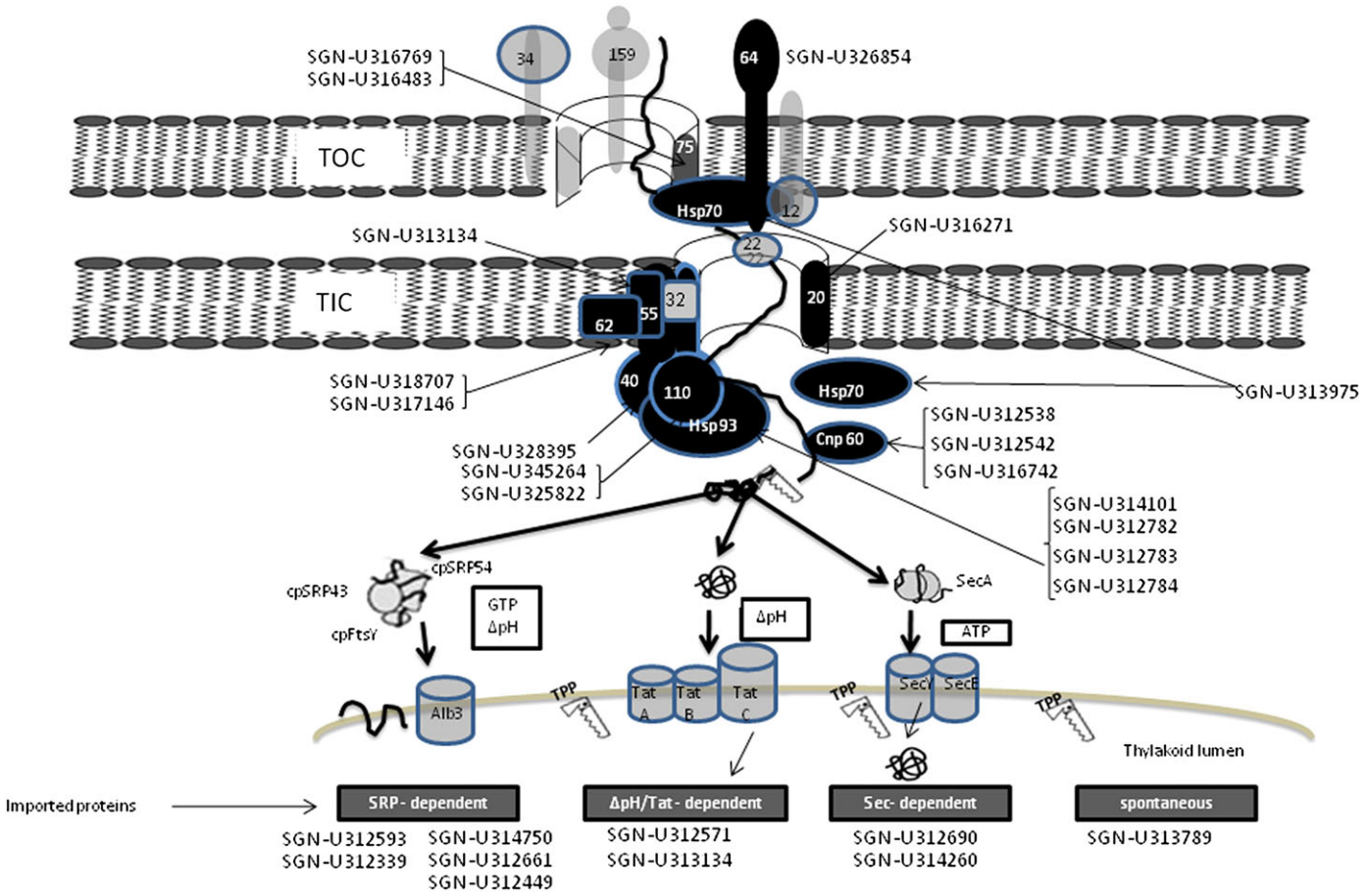

Fig. 5. Schematic representation of the plastidial protein import system with mention of proteins encountered in the tomato chromoplast proteome. Note the presence of most of the Tic proteins and the absence of most of the Toc proteins and of all thylakoid import machinery. However, a number of proteins known to be transferred to the lumen by the thylakoid import system are present. Proteins are represented by their unigene SGN code.

involved in these pathways was identified, probably as a result of the loss of thylakoid structure. Nevertheless, a number of proteins known to be translocated to the lumen (Klösgen *et al.*, 2004) are present (Fig. 5).

The Chloroplast Envelope Quinone Oxidoreductase CeQORH has been mentioned as being imported through a non-canonical signal peptide transport by Miras *et al.* (2002). Intracellular vesicular transport has been observed in chloroplasts by electron microscopy and the use of effectors of vesicle formation (Westphal *et al.*, 2001). Based on predictions made by bioinformatic analysis of the *Arabidopsis* genome, Andersson and Sandelius (2004) underlined the likely presence in chromoplasts of 33 *Arabidopsis* homologues of yeast vesicular trafficking components, among which five were detected in the tomato chromoplast proteome (listed in 'Vesicular transport' in Table 2).

Conclusions

The present study reveals a number of important characteristics of the non-photosynthetic plastid, the tomato chromoplast. A total of around 1000 chromoplast proteins has been reported in tomato. Whilst the predicted size of the plastid proteome of *Arabidopsis* varies between 1900–2500 proteins (Abdallah *et al.*, 2000), 2700 proteins (Millar *et al.*, 2006), and 3800 proteins (Kleffman *et al.*, 2004), the actual number of proteins reported is 1280 (Zybailov *et al.* 2008). In a study of the pepper chromoplastic proteome, a total of 151 proteins were recorded (Siddique *et al.*, 2006). Anyway, it is clear that the list of plastidial proteins does not represent the whole predicted proteome of the organelle, although uncertainties in the predictions do not allow us to determine the precise coverage of the total proteome. There are several reasons for the limited coverage of the plastidial proteome observed for the tomato chromoplast as well as for other plastids. The number of proteins probably varies according to the development stage of the plastids and environmental conditions. Also, the extraction procedures employed do not yield all the membrane-embedded proteins and low-level soluble proteins. Finally, many proteins are probably present at levels that cannot be detected by the current technologies of separation and sequencing, although the modern Qtrap technology used here can generally detect femtomole levels

(10^{-15}). Another consideration that must be taken into account is that proteomic data, similarly to transcriptomic data, are not necessarily indicative of actual metabolic or regulatory activities. Parallel enzymological, metabolomic, and fluxomic studies are necessary to assess fully the metabolic activity of the organelle. Nevertheless, proteomic data does give useful information for genome annotation and subcellular localization of proteins. Furthermore, when a whole set of proteins of a specific metabolic pathway is identified, proteomic analysis can give relevant biological information. Out of 325 thylakoid proteins described by various authors in chloroplasts (Peltier *et al.*, 2002, 2004; Giacomelli *et al.*, 2006; Rutschow *et al.*, 2008) 119 have been found in our fractions. These proteins are not involved in a specific pathway; but take part in a variety of processes such as protein degradation, photosynthesis, hormone metabolism etc. Out of these, 23 were found in plastoglobuli (Vidi *et al.*, 2006; Ytterberg *et al.*, 2006) with 14 involved in photosynthesis. Another interesting characteristic of the chromoplast is the total absence of the thylakoid protein transport machinery. An additional observation is the low number of proteins involved in photosynthesis, with only 22% and 39% of the proteins of PSI and PSII, respectively. This is related to the absence of chlorophyll and photosynthetic activity associated with the presence in the chromoplast of active chlorophyll catabolism and autophagy of photosynthetic proteins. On the other hand, the presence of all the Calvin cycle proteins is striking, including all Rubisco subunits and other proteins required for activity. This could be related to the recycling of CO_2 produced by the oxidative pentose phosphate pathway. Another major feature is the capacity for lipid biosynthesis, which is attested by the presence of all the proteins involved in the synthesis of 3-oxoacyl-ACP, the precursor of fatty acids, including all acetyl-CoA-carboxylase monomers. In conclusion, the chromoplast proteome analysis carried out in the present study allows us to gain new insights into the complexity of the functioning of this particular organelle.

Supplementary data

The following supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Status of the tetrapyrrole biosynthetic pathway in tomato chromoplasts.

Supplementary Fig. S2. Lipid biosynthesis pathway showing the presence of proteins encountered in the tomato chromoplastic proteome.

Supplementary Fig. S3. MS/MS spectra for 36 proteins identified with only one amino acid sequence.

Supplementary Table S1. Functionnal classification, predictions of localization, and comparison with other plastid proteomes of the 988 proteins of the tomato chromoplast proteome.

Supplementary Table S2. Identification of the tomato chromoplast proteome by LC-MS/MS analysis using a Q-Trap mass spectrometer.

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