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The proteOMIC era: a useful tool to gain deeper insights into plastid physiology

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Abstract Chloroplasts, the green plastid-type present in all photosynthetic organisms, are the physical place where photosynthesis and many other metabolic pathways occur. Chloroplasts are essential for plants, not only by performing photosynthesis but also due to the production of important compounds comprising a great variety of secondary metabolites, lipids, and plant hormones. The production of these compounds is highly regulated and coordinated with development in crops that have fruits with specialized plastids called chromoplasts. Study of plastid biology is essential to understand plant physiology and how plastid biogenesis and development impact plant growth. With the introduction of the genetic and genomic technologies to plant research the discovery and functional characterization of chloroplast proteins was boosted. Nowadays, technologies such as transcriptomics and proteomics are routinely used to assign functions to chloroplast proteins. The generation of high-throughput data sets allows a great increase in our knowledge about many chloroplast processes (e.g. pigment synthesis and accumulation, chloroplast to chromoplast transition, protein degradation) but also the possibility to apply these knowledge to genetically modify plants to improve beneficial traits (e.g. biomass, carotenoid content). The aim of this review is to highlight the importance of proteomic approaches for the study of plastid biology and how this technique speeds up the gain of knowledge in this field.

Keywords Proteomics · Chloroplast · Chromoplast · Carotenoids · Protein degradation · Clp protease · Development · Tomato · Arabidopsis

1 Introduction

In plants, several types of plastids such as, proplastids (progenitors of all types of plastids), leucoplasts (colorless plastids involved in storage of lipids, starch and proteins), chromoplasts (colored plastids due to the accumulation of carotenoids) and chloroplasts are found (Pogson et al. 2015). From those, the chloroplast is the characteristic organelle of plants and green algae. Chloroplasts are descendants of an ancient cyanobacterial endosymbiont and they harbour its own genome (∼ 120–135 genes), which make them a semiautonomous organelle (Green 2011). Even though many of their functions are conserved, most of the chloroplast genes were transferred to the host nucleus during subsequent evolution of this organelle (Timmis et al. 2004). In this organelle one of the most important biological processes in earth takes place—photosynthesis. However, this is not the only important process occurring in this organelle. Chloroplasts

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are indispensable for plant development due to the fact that essential biochemical processes such as synthesis of isoprenoids (e.g. pigments), lipids and plant hormones take place inside this compartment. Due to this the chloroplast requires a highly tight regulation of all these processes through its development. Generally, chloroplasts develop from undeveloped proplastids, which contain vesicles but yet undifferentiated structures. During this differentiation thylakoids are formed and stacked into defined grana (Pogson et al. 2015). The thylakoid membrane is an internal lipid membrane which contains protein complexes and together provides the platform for the light reaction of photosynthesis (Pribil et al. 2014). Besides thylakoids, chloroplasts count with a double membrane system, the outer and inner envelope, which is a phospholipid bilayer. Chloroplasts also have the stroma which is especially rich in proteins and enzymes essential for vital cellular processes. In addition, stroma also contains DNA, ribosomes and other molecules required for protein synthesis. Altogether chloroplast compartments possess a repertoire of $\sim$ 2000-3000 proteins, being mostly encoded in the nucleus and posttranslationally allocated in the organelle to fulfil numerous functions such as participation in photosynthesis machinery (structural and functional) and metabolic pathways (Jarvis and Lopez-Juez 2013). This requires a highly-coordinated communication between chloroplast and nucleus through all stages of the organelle development. For instance, stoichiometric assembly of nuclear- and plastidic-encoded proteins with chlorophylls and carotenoids is facilitated through the coordination of protein transcription, translation, import and turnover. This contributes to the limitation of oxidative damage caused by free photoreactive pigments and to assure optimal rates for photosynthesis (Pogson et al. 2015). In addition, synthesis and import of metabolites within the chloroplast are essential for many vital processes as for example thylakoid formation. Furthermore, proper function of chloroplast requires a precise signalling network which includes inter-organelle communication between nucleus and chloroplast (for coordination of protein synthesis) and between chloroplasts, peroxisomes and mitochondria for metabolite exchange (Jarvis and Lopez-Juez 2013; Pogson et al. 2015). Protein import (mainly TIC and TOC complexes) also plays a key role in chloroplast function and development due to the import of nuclear-encoded proteins harbouring an amino-terminal cleavable targeting signal for further assembly of multiprotein complexes inside the chloroplast (Li and Chiu 2010; Inoue et al. 2013).

Considering the different levels of regulation/communication/compartmentalization with other organelles (and in the chloroplast) plus synchronization with organelle biogenesis and plant development, it is clear that the study of chloroplast biology/physiology it is not an easy task. Early functional studies of chloroplasts relied on the use of biochemical and biophysical approaches. During 80 s and 90 s, chloroplast DNA transformation was developed as well as strategies to disrupt nuclear genes by inserting transposons or T-DNAs (Armbruster et al. 2011). These technological advances immensely contributed to the study of chloroplast function. More recently, with the sequencing of entire genomes and the establishment of high-throughput methodologies for gene expression analysis, chloroplast research entered to the genomic era (Leister 2003). In the last decade, with the peak of functional genomics (analysis of transcriptome, proteome and metabolome) chloroplast research started to explore (and continues exploring) new chloroplast functions by the characterization of new pathways and new components of already known pathways. In addition, this also facilitates the study of molecular mechanisms explaining abnormal phenotypes in available mutants (Table 1). The focus of this review is to discuss important discoveries of chloroplast functions/responses by means of proteomic approaches representing an important contribution to the study of chloroplast biology and plant physiology.

2 Plastid proteomics: from chloroplast isolation to mass spectrometry

In order to obtain high quality results from proteomic experiments using plant organelles, it is extremely important to isolate the organelle of interest (e.g. chromoplast) and determine their purity. Isolation of chromoplasts has been reported by various methods such as sucrose, percoll and Nycodenz gradients (Fig. 1 (Tetlow et al. 2003; Barsan et al. 2010; Zeng et al. 2011b). One of the most commonly used methods is the sucrose gradient. This method is applied on a crude extract of tomato chromoplast which is prepared from around 300 g of tomato fruits

Table 1 Summary of proteomic strategies that contributed with relevant discoveries in the field of plastid physiology

Plant/alga model	Methodology	Relevance	Reference
Tomato	MALDI-ToF/LC–MS	Characterization of chromoplast biogenesis	Faurobert et al. (2007)
Tomato	(1D)nanoLC–MS/MS (spectral counting)	Proteome analysis of chloroplast to chromoplast transition	Barsan et al. (2010) Barsan et al. (2012)
Tomato Micro-Tom (cv. *Black and White Beauty*)	(2-D)LIT-ToF MS	Detailed analysis of the Micro-Tom proteome in two different varieties with different carotenoid content	Suzuki et al. (2015)
Tomato	LC–MS/MS (iTRAQ)	Analysis of the importance of BZR1 transcription factor in fruit ripening	Liu et al. (2016)
Tomato/carrot/pepper/ watermelon/papaya/ cauliflower	(1D) nanoLC–MS/MS (Synapt HDMS)	Commonalities and differences of six different plant plastid proteomes	Wang et al. (2013)
Chlamydomonas	LC–MS/MS (quantitative shotgun proteomics with ^{15}N-labeled standard)	Discovery of a chloroplast unfolded response	Ramundo et al. (2014)
Arabidopsis	(1D) LC–MS/MS	Discovery of ClpS1 substrates and its relevance for plant physiology	Nishimura et al. (2013)
Arabidopsis	(1D) LC–MS/MS	Discovery of a new plant specific substrate selector ClpF	Nishimura et al. (2015)
Tobacco	LC–MS/MS (in solution digestion)	Short list with promising Clp substrates	Moreno et al. (2018)
Arabidopsis	LC–MS/MS (spectral counting)	Precise localization of enzymes involved in carotenoid and prenylquinones pathways	Joyard et al. (2009)
Red-flesh sweet orange	(2D) MALDI-ToF-ToF/LC-MS	Provide new insights into molecular processes regulating lycopene accumulation in the red-flesh sweet orange	Pan et al. (2012)
Potato	LC-MS/MS (iTRAQ)	Regulation of carotenoid sequestration capacity is an important mechanism by which carotenoid stability is regulated	Li et al. (2012)
Tomato	MALDI-ToF/ToF MS/MS	Increase in CHRC levels may contribute to the enhanced carotenoid content in these high-pigment fruits by assisting in the sequestration and stabilization of carotenoids	Kilambi et al. (2013)
Rice	(2D) MALDI-ToF MS/MS	Metabolic adaptation process of transgenic golden rice with enhanced carotenoid content	Gayen et al. (2016)

subjected to a series of washes, grinding, filtering, and centrifugation steps (Barsan et al. 2017). The crude chromoplast suspension is layered on top of the sucrose gradient and subjected to centrifugation allowing the different organelles to be localized in the different fractions (e.g. fraction 2 and 3 contain

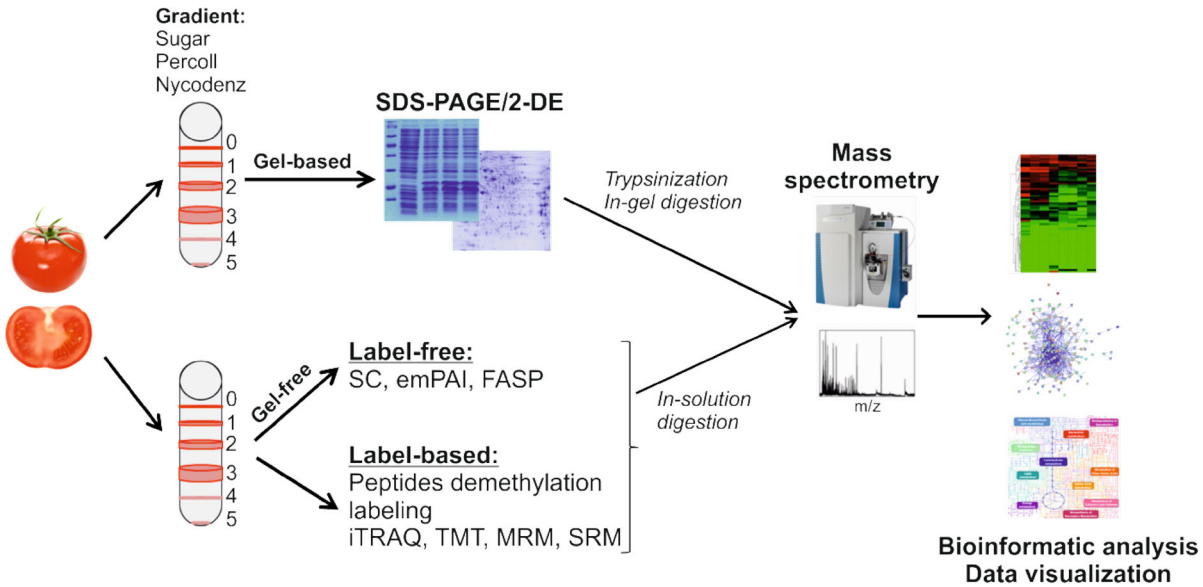

Fig. 1 Methodologies to perform plastid proteomics. Chromoplasts are isolated by different techniques using gradient centrifugation (e.g. sugar, percoll, Nycodenz). Fractionated chromoplasts are found in bands 2 and 3. These fractions are loaded in the gel (gel-based approach) and bands are cut for further trypsin digestion. Gel-free methodologies include label-free techniques such as SC, emPAI, and FASP, but also label-based techniques such as iTRAQ, TMT, MRM and SRM. In this case, peptides are digested in a solution. Peptides generated from trypsin digestion are measured in a mass spectrometer (LC–MS/MS). Results are normalized and analysed with different software's/servers (e.g. MASCOT). Data can be visualized as heat maps, as a protein network or as a metabolic pathway. *SDS–PAGE* sodium dodecyl sulfate–polyacrylamide gel electrophoresis, *SC* spectral counting, *FASP* filter-aid sample preparation, *emPAI* exponentially modified protein abundance index, *iTRAQ* isobaric tags for relative and absolute quantification, *TMT* tandem mass tag, *MRM* multiple reaction monitoring, *SRM* selected reaction monitoring

chromoplasts; Fig. 1). Fractions of interest are recovered and once the intact chromoplasts are isolated different approaches such as microscopy, MS–MS shotgun proteomics, enzymatic activities of marker enzymes (e.g. glyceraldehyde 3-phosphate dehydrogenase for plastids, cytochrome-c oxidase for mitochondria, catalase for peroxisomes and lactate dehydrogenase for cytoplasm) and western blot analysis (using polyclonal antibodies against various organelle markers) can be used to confirm the purity of the isolation. After obtaining highly-pure chromoplasts different approaches, prior protein digestion and mass spectrometry, can be performed. Gel-based techniques such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional electrophoresis (2-DE) can be used to run the isolated chromoplast samples to extract the bands or dots, respectively, and proceed with the protein digestion (Fig. 1). The most commonly used protocol is the trypsin digestion which allows the generation of thousands of peptides for further quantification by mass spectrometry (Faurobert et al. 2007; Barsan et al. 2010; Suzuki et al. 2015). There are also gel-free approaches such as label-free and label-based techniques. On one hand, label-free techniques include spectral counting (SC), exponentially modified protein abundance index (emPAI) and filter aid sample preparation (FASP), where there is no need of a tag for the samples (Wisniewski et al. 2009; Li et al. 2017). On the other hand, label-based techniques such as peptides demethylation labelling, isobaric tags for relative and absolute quantification (iTRAQ), tandem mass tag (TMT), multiple reaction monitoring (MRM), and selected reaction monitoring (SRM) are used to perform absolute quantification of proteins employing tags (Martinez-Esteso et al. 2011; Li et al. 2013; Song et al. 2015a, b; Zeng et al. 2015). Different mass spectrometers, such as BiFlex III MALDI-TOF (Bruker Daltonics), LTQ-Orbitrap (Thermo Fisher Scientific) or hybrid quadrupole Orbitrap (Q-exactive; Thermo Fisher Scientific) can be used to process the samples (Faurobert et al. 2007; Barsan et al.

2010, 2012; Liu et al. 2016). Once samples are processed the obtained chromatograms are analysed using software's such as MASCOT or MaxQuant (Barsan et al. 2012; Kosmacz et al. 2018) leading to quantification and sample normalization. In order to obtain an extensive protein annotation comparison of the results with different databases can be done. Finally, the data can be subjected to different statistical tests (e.g. one-way ANOVA, two-ways ANOVA, student *t* test) according to the experimental set up (e.g. time points, treatments, induction). The data can be presented or visualized in different ways (e.g. heat map, protein networks, protein pathways) using available online tools (TAIR, plant proteome database, Map-Man; Fig. 1).

3 Plastid biogenesis and development

3.1 Chloroplast to chromoplast differentiation

As mentioned above proplastids can differentiate to several types of plastids (e.g. chloroplast and chromoplast; Fig. 2). One of those, are the chromoplasts, which are non-photosynthetic plastids found in flowers and fruits, and occasionally in roots and leaves. The fruit is a very interesting model of study due to a phenomenon called ripening. Ripening involves a series of biochemical and physiological events that result in organoleptic changes in texture, aroma and colour (Barsan et al. 2010). During ripening the most important and visible changes correspond to the loss of chlorophyll and the synthesis of carotenoids which provides, for instance, red or orange colour to the fruit (Fig. 2). In this process proplastids (and chloroplasts) differentiate to chromoplasts, the most abundant type of plastids in the fruit after ripening (Fig. 2). At early stages of the proteomic era all the efforts were placed in the proteome of chloroplast but recently more and more reports have been published about chromoplast proteomics in order to understand the physiology of the fruit by characterization of plastid differentiation during fruit ripening (Barsan et al. 2010, 2012; Wang et al. 2013; Suzuki et al. 2015). One of the first reports using proteomics to characterize chromoplast biogenesis during tomato (cherry) development is the work published by Faurobert et al. (Faurobert et al. 2007). In this work 90 proteins were identified either by matrix-assisted laser-desorption ionization time-of-flight peptide mass fingerprinting or liquid chromatography-mass spectrometry sequencing and expressed sequence tag database searching. Interestingly the proteins found here clustered in different processes such as amino acid metabolism and protein synthesis, and were mainly expressed during cell division stage. During cell expansion phase, proteins involved in photosynthesis and cell wall formation transiently increased due to the requirements in the early stage of the development of the fruit which it is still green and just starting to grow. In contrast, the majority of proteins involved in carbon compounds and carbohydrate metabolism or oxidative processes were up-regulated during fruit development showing their peak in the mature fruit. In line with the development of the fruit, proteins linked with stress responses and fruit senescence showed their peak in the mature fruit as well. This study was complemented by Barsan et al. (Barsan et al. 2010) where they showed that proteins of lipid metabolism and trafficking were very abundant, including all the proteins of the lipoxygenase pathway required for the synthesis of lipid-derived aroma volatiles. In addition, chromoplast samples (red fruit) analyzed in this study lacked proteins of the chlorophyll biosynthesis branch and contained instead proteins involved in chlorophyll degradation. Some of these findings were corroborated and extended by Barsan et al. (Barsan et al. 2012) who studied the transition of chloroplast to chromoplast in tomato fruit (mature-green, breaker and red stages). With more advanced mass spectrometry technology for protein identification and quantification, they were able to quantify 1529 proteins and perform validation of six (from different metabolic pathways) by western blot analysis. The main two findings of this study include metabolic shifts that appear to be associated with chromoplastogenesis (formation of the chromoplast). The first important finding, observed mostly at the stage between breaker and red fruit is a strong decrease in abundance of proteins of light reactions (photosynthesis and photorespiration) and carbohydrate metabolism (starch synthesis/degradation). This is in line with the loss of chlorophyll in the fruit and therefore the absence of photosynthesis or components of the photosynthetic apparatus or associated reactions. The second finding is an increase in terpenoids biosynthesis (including carotenoids) and stress-response proteins (e.g. ascorbate–glutathione, abiotic stress, redox, heat shock) (Barsan et al. 2012). This is preceded by

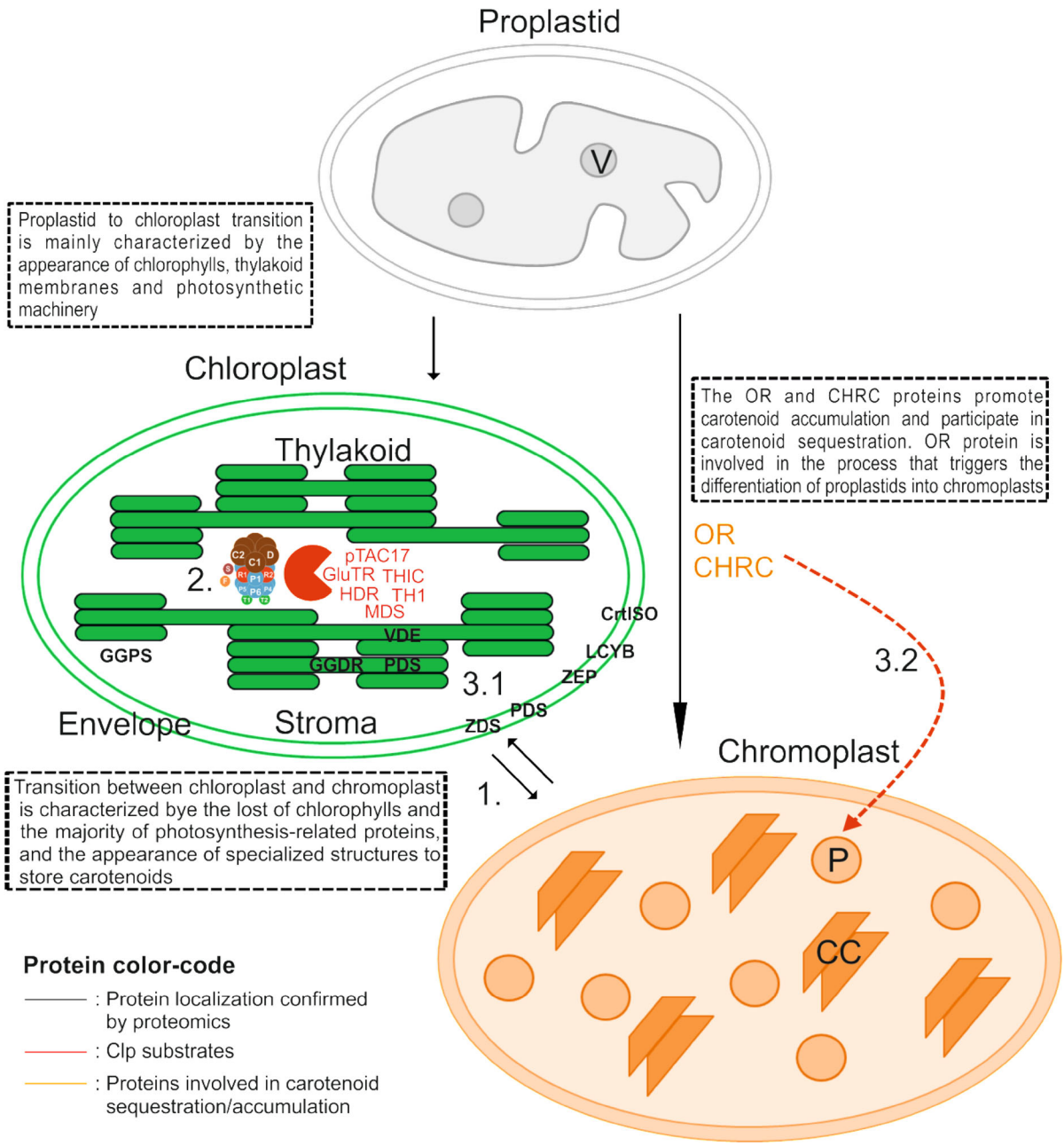

Fig. 2 Schematic representation of changes occurring during proplastid differentiation and key biological processes characterized by proteomic analysis. Proplastids, which are undifferentiated plastids, can give rise to chloroplast or chromoplast, amongst other type of plastids. In (1.) the transition from chloroplast to chromoplast is shown. This is mainly characterized by the loss of chlorophyll and thylakoid structures (and therefore the majority of protein subunits of the photosystems) and the appearance of plastoglobules (P) and carotenoid crystals (CC). In (2.) the Clp protease located in the stroma is shown (with the different subunits). In red confirmed and/or promising candidate substrates are shown. In (3.1) the location of different enzymes of the MEP and carotenoid pathway detected through proteomics are shown. In (3.2) the major role of OR and CHRC proteins in carotenoid sequestration in plastoglobules is depicted. *V* vesicle, *GGPS* geranyl geranyl pyrophosphate synthase, *GGDR* geranyl reductase, *PDS* phytoene desaturase, *VDE* violaxanthin deepoxidase, *ZDS* zeta-carotene desaturase, *ZEP* zeaxanthin epoxidase, *CrtISO* carotenoid isomerase, *LCYB* lycopene β-cyclase

the accumulation of plastid-encoded acetyl Coenzyme A carboxylase D proteins which are involved in providing a storage matrix that will accumulate carotenoids (lycopene is the main pigment accumulated in the tomato fruit). The structural differentiation of the chromoplast is characterized by a sharp and continuous decrease of thylakoidal proteins whereas envelope and stroma proteins remain stable (Barsan et al. 2012). This correlates with the disruption of the photosynthetic machinery for thylakoids and photosystem biogenesis and the loss of the plastid division machinery. A more recent and extensive analysis conducted by Suzuki et al. (Suzuki et al. 2015) in which they compared four distinct stages of Micro-Tom fruit development and two different varieties—Black and White Beauty provided new insights into specific proteins related with tomato fruit development. Interestingly, they could confirm that proteins related to photosynthesis remain through the orange maturity stage of Micro-Tom while thylakoids showed to no longer exist in this stage suggesting that at least some morphological changes occur before the protein content change (Suzuki et al. 2015). Moreover, specific proteins involved in accumulation of carotenoids, fruit colour and the differentiation of chromoplast were identified. These proteins are CHRC (plastid-lipid-associated protein) and HrBP1 (hairpin binding protein-1), both showed a decrease in the proteomic analysis performed in the Black and White Beauty varieties which could explain the lower carotenoid content present in these varieties compared to Micro-Tom.

Proteomic have been also used to study the impact of salinity in fruit development of crop plants. Specifically, in tomato the varieties Cervil and Levovil were used to evaluate the effect of salinity and calcium treatment in tomato fruit. Most of the identified proteins found in both treatments (Na or Ca + Na) are involved in carbon and energy metabolism, salt and oxidative stress and proteins associated with ripening process. In general, a large variation on protein abundance was found for the two genotypes which in turn might be correlated with the salt treatment or/and fruit ripening stage (Manaa et al. 2013). The greatest effect observed in this report was the protective properties of calcium which showed to limit the impact of salinization on metabolism and ripening process but also induced plant salt tolerance. On the other hand, proteomic studies are also useful to gain insights into molecular mechanisms. One example is the work published by Liu et al. (Liu et al. 2016) in which by using over expressor lines of BZR1-1D, an important transcription factor involved in fruit development in tomato, they provided new evidence revealing the molecular link between brassinosteroids (BR) signalling pathway and downstream components involved in multiple ripening-associated events during tomato fruit ripening. Using relative and absolute quantitation (iTRAQ) labelling technology the authors found 411 differentially expressed proteins implicated in different processes such as, light reaction, plant hormone pathways, and cell-wall-related metabolism (Liu et al. 2016). Considering that the light reaction metabolic pathway was identified as a markedly enhanced pathway by BZR1 transcription factor during tomato fruit ripening, a deeper look into promising proteins in this pathway was performed. Data analysis revealed a probable 2-oxoglutarate-dependent dioxygenase (2-ODD2) protein, which is involved in gibberellin (GA) biosynthesis, to be a promising candidate. 2-ODD2 showed a significant increase in all four developmental and ripening stages analysed in the study. Due to the cross-talk between BR and GA this protein could be the link between the hormonal cross-talk and fruit development.

The robustness of the results of the above-mentioned strategies is exposed in a previous report (Wang et al. 2013) where the authors analysed protein composition of chromoplast of six different crop species (carrot, orange cauliflower, tomato, red papaya, watermelon and red bell pepper). A large number of proteins were identified across the six crop species (953–2262) where about 60% where localized to the plastid. These proteins were involved in protein metabolism, transport, amino acid metabolism, lipid metabolism and redox which are consistent whit previous results obtained from studies on tomato (Faurobert et al. 2007; Barsan et al. 2010, 2012). Interestingly, each of the species also has specific changes in their proteome due to specific proteins they possess in their proteomes (e.g. capsanthin/capsorubin synthase and fibrillins in pepper).

Taken together the above-mentioned evidences, it is quite clear that with the development of mass spectrometry techniques a great contribution in understanding the chromoplast proteome was done. However, there is still ongoing research to integrate all these findings together with physiological data that

could provide a more robust interpretation of the metabolic and physiological status of the plant.

4 Plastid proteolysis

4.1 Clp protease: an essential protease to shape plastid differentiation and plant development

Protein degradation is an essential process occurring inside every living organism on earth. This process helps to maintain protein homeostasis in the cell. The main key players in this process are special proteins called proteases. In plastids, the major stromal protease is the caseinolytic protease (Clp). Clp is one (if not the most) of the most studied plastidial protease in the last 20 years. As mentioned above plastids change their morphology in response to environmental stimuli and developmental transitions. Therefore, dynamical adjustment of the plastid proteome by controlling the abundance of both nucleus-encoded and plastid-encoded proteins is essential. Proteases play an important role in the interconversion of plastid types and the maintenance of plastid homeostasis (Kato and Sakamoto 2010). Biochemical and genetic studies have unravelled several proteolytic activities in plastids (Sakamoto 2006). However, due to novel proteomic experimental design with the major advances in proteomics more proteases, protease interactors and processes tightly related with proteolysis and chloroplast biogenesis/development have been recently discovered (Ramundo et al. 2014; Ramundo and Rochaix 2014; Kim et al. 2015; Nishimura et al. 2015; Moreno et al. 2018). In plants, the Clp protease represents the most abundant and complex stromal protease system (Olinares et al. 2011). Clp is believed to play a central role in protein quality control as a housekeeping protease (Clarke et al. 2005). As a complex, Clp protease is composed of two heptameric rings, the P-ring (ClpP3, ClpP4, ClpP5 and ClpP6), with proteolytic activity, and the R-ring (ClpR1, ClpR2, ClpR3, ClpR4 and ClpP1). The R subunits of the R-ring lack the catalytic activity which instead is carried by ClpP1, the only subunit encoded in the plastid genome. Other components of this protease are the chaperone ring (ClpD and ClpC), the adaptor proteins (ClpS and ClpF) and the accessory proteins (ClpT1 and ClpT2). In the first decade of Clp protease research specific functions of the majority of its subunits were elucidated through genetic studies using RNAi and null mutants in Arabidopsis (Kim et al. 2001; Peltier et al. 2001; Zheng et al. 2002; Peltier et al. 2004; Sjogren et al. 2004; Rudella et al. 2006; Sjogren et al. 2006; Zheng et al. 2006). The following years, great efforts were made to study the pale-green or variegated phenotypes obtained in the majority of the RNAi or null mutants in Arabidopsis leading to employment of proteomic strategies to tackle important aspects such as plastid development, Clp stoichiometry and one of the biggest challenge the Clp protease substrates (Koussevitzky et al. 2007; Kim et al. 2009; Zybailov et al. 2009). In these reports, molecular characterization of different Clp subunits (mostly from P- and R-rings) contributed to the field with specific functions for each subunit. Moreover, with the use of proteomics, it was possible to show that processes such as photosynthesis, protein import, proteolysis, chaperone networks, among others, were tightly connected with Clp expression. These were the first reports attempting the identification of Clp substrates. However, the use of the above-mentioned mutants (pale green and variegated phenotypes) and the pleiotropic effects accumulated in those plants made researchers doubt the veracity of the proposed targets. Nevertheless, a big number of processes affected by the repression of the Clp protease (e.g. plastid biogenesis and development, photosynthesis and protein import) were identified in these mutants. The connection between these processes and the Clp protease contributed to the understanding of the importance of this protease for plant physiology and development. Taking advantage of the advances in proteomics, the precise stoichiometry of the Clp protease was resolved (Olinares et al. 2011). The stoichiometry of 1:2:3:1 for ClpP3:ClpP4:ClpP5:ClpP6 and 1:1:1:1:3 for ClpR1:ClpR2:ClpR3:ClpR4:ClpP1, determined by mass spectrometry-based absolute quantification using stable isotope-labelled proteotypic peptides, revealed the importance of each subunit.

In the last 5 years, the advances of proteomics combined with very elegant experimental designs allowed great discoveries in the field of protease substrates. Ramundo et al. developed a repressible system, based on the vitamin-sensitive Thi4 riboswitch (Ramundo et al. 2013), to decrease gene expression of plastid genes and used it to induce the repression of the Clp protease in *Chlamydomonas*

reindhartii. This strategy avoided any accumulation of pleiotropic effects in the mutant (Ramundo et al. 2014). By gradual reduction of Clp expression, authors identified direct effects such as alteration in chloroplast morphology, formation of vesicles and extensive induction of cytoplasmic vacuolization (reminiscent of autophagy). Analysis of transcriptome and proteome suggested a set of proteins that could be promising substrates for the Clp protease. Those proteins are involved in chloroplast metabolism, protein synthesis/turnover/folding, and DNA recombination. Interestingly, the specific increase in accumulation (both at the RNA and protein level) of small heat shock proteins, chaperones, proteases, and proteins involved in thylakoid maintenance upon perturbation of plastid protein homeostasis suggests the existence of a chloroplast-to-nucleus signalling pathway involved in organelle quality control. This suggests that response of a chloroplast unfolded protein is conceptually similar to the one observed in the endoplasmic reticulum and in mitochondria. Other breakthrough in the Clp protease field was the identification and characterization of the substrate locator ClpS1 by Nishimura et al. (Nishimura et al. 2013). In this work, authors determined the physiological significance of ClpS1 in the chloroplast by using quantitative comparative proteomics of a null ClpS mutant (*clps1*) and comparison to a ClpC chaperone null mutant (*clpc1-1*). Proteomic results showed only slight changes at the *clps1* mutant proteome. A clear significant down-regulation was observed for tetrapyrrole metabolism (18%; $P < 0.05$) and three Map-Man bin functions (starch metabolism, glycolysis and amino acid metabolism) were only mildly reduced ($P < 0.1$). In this analysis 39 up-regulated and 36 down-regulated proteins were identified. This pointed into the 39 up-regulated proteins as a set of promising candidates to be ClpS1 substrates. For the confirmation, authors developed an affinity-based strategy using recombinant GST-ClpS1 fusion protein coupled to mass spectrometry. Finally, they found five very promising candidate substrates for ClpS1 (glutamyl-tRNA reductase/GluTR, chorismate synthase/CS, PRLI-interacting factor, pTAC17, 3-Deoxy-D-arabinoheptulosonate 7-phosphate synthetase/DHS, and pyridox oxidase domain protein, PYROX). From those, further validation of three of them (due to availability of the antibodies) showed that only GluTR and pTAC but not the uvrB/uvrC motif-containing protein (UVR; identified as putative substrate in the affinity enrichment experiment using chloroplast stroma from *clps::clpc1* double mutant) are true ClpS1 substrates (Fig. 2). This was further proved and characterized in detail by Apitz et al. (Apitz et al. 2016) for GluTR. In this work they demonstrated that proteolytic activity of Clp protease counteracts GluTR-binding protein binding to assure the appropriate content of GluTR and the adequate ALA synthesis for chlorophyll and heme in higher plants. In addition, a new substrate selector was identified for the Clp system, ClpF, and its interaction with ClpS1 was confirmed (Nishimura et al. 2015). By using MS/MS analysis combined with the GST-tag, used previously for the identification of the ClpS1 substrates, authors demonstrated that ClpF interacts with ClpS1 via its N-terminal domain (NTD). These evidences allowed the construction of a model in which either ClpS1 or ClpF could recognize different types of Clp substrates but also they can act together to recognize other type of substrates (Nishimura et al. 2015). These findings represented a great progress in the protease field because allows the study of protein sequences that are recognized by the substrate locators ClpS1 and ClpF. Having this knowledge, it is possible to manipulate accumulation level of any protein of interest by modifying recognition sites in their protein sequences leading to improvement of their expression in any heterologous system where these substrate locators are present.

Most of the evidences gathered for the Clp protease comes from studies in *Arabidopsis thaliana* as exposed above. However, recently a novel strategy to identify protease substrates in tobacco was reported (Moreno et al. 2018). In this work, authors designed a very interesting strategy where they employed an ethanol induction system to repress the expression of three different subunits of the Clp protease (ClpP6, ClpC and ClpT1-T2) and followed changes in their proteome through time. With the time-resolved proteomic strategy it was possible to avoid the pleiotropic effects present in constitutive knock out/knock down mutants leading to identification of true substrates upon ethanol induction and repression of different Clp subunits. This experimental design showed 11 very promising candidates, that passed a very strict cut-off (increase in abundance at early time-points (24 h) and $> 50\%$ increase in protein abundance), involved in MEP pathway (2-C-methyl-D-erythritol 2,4-

cyclodiphosphate synthase, MCS, and 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase, HDR), photosynthesis (NDHM and ATPD), ribosome (plastid ribosomal protein, PSPR2, and ribosomal protein 1, RPS1), thiamin biosynthesis (TH1 and THIC), amino acid metabolism (ARGH), translation (pTAC12) and in carbon fixation (ferredoxin thioredoxin A, FRTA) (Fig. 2). Despite the efforts of authors to validate the substrate list via western blot experiments, the lack of good-working antibodies in tobacco impede further analysis of protein accumulation in the tested time points. Nevertheless, this approach opens new possibilities for high-throughput substrate characterization of proteases and further validation via western blot or affinity purification experiments.

5 Metabolic pathways

5.1 Carotenoid pathway

Carotenoids are C40 isoprenoid compounds rich in double conjugated bonds in their structure. This allows a great variety of functions, such as multiple antioxidant activities, including the ability to scavenge singlet oxygen and peroxyl radicals (Krinsky 1989; Hirschberg 2001). In plants, carotenoids are not only precursors of phytohormones (e.g. abscisic acid, ABA, and strigolactones, SLs) and signaling molecules (e.g. β-cyclocitral/β-cc, and dihydroactinidiolide/dhA) but they also play roles in photosynthesis, photomorphogenesis, photoprotection and development (Dall'Osto et al. 2007; Ramel et al. 2012; Lv et al. 2015; Nisar et al. 2015). Carotenoid synthesis starts with the formation of phytoene by the action of the enzyme phytoene synthase (PSY). The PDS (phytoene desaturase), ZDS (ζ carotene desaturase), CRTISO (carotenoid isomerase), and ζ-ISO (ζ-carotene isomerase) enzymes convert phytoene to lycopene via ζ-carotene. Cyclization of lycopene marks a branching point in the pathway: one branch leads to β-carotene and its derivatives (zeaxanthin and violaxanthin), whereas the other leads to α-carotene and lutein (Hirschberg 2001). Due to the fact that carotenoid pathway is mainly regulated at transcriptional level not many attention has been drawn to protein studies. With the recent advances of proteomic techniques valuable information regarding protein localization and protein functions participating in key processes influencing carotenoid accumulation has been obtained. The first contribution of a proteomic strategy was the use of a semi-quantitative proteomic approach (spectral counting) to identify the precise localization of the biosynthesis of carotenoids (and other isoprenoids) within the chloroplast (e.g. in envelope membranes, stroma, and/or thylakoids) (Joyard et al. 2009). The results showed that the majority of carotenoid enzymes are located in the envelope membrane (PDS, ZDS, LYC, carotene β-ring hydroxylase, β-hydroxylase 1, ε-hydroxylase, zeaxanthin epoxidase, antheraxanthin epoxidase, neoxanthin synthase and 9-cis-epoxycarotenoids dioxygenase) or in the thylakoid membrane (PDS, zeaxanthin de-epoxidase, antheraxanthin de-epoxidase, zeaxanthin epoxidase and antheraxanthin epoxidase) but also some showed no compartmentalization data (β-hydroxylase 2 and lycopene ε-cyclase) (Joyard et al. 2009) (Fig. 2). This study opened new possibilities to use proteomics to find new protein functions and multiple processes supporting carotenoid accumulation and production. One of the first proteomic studies using proteomic approaches to characterize carotenoid accumulation was performed in red-flesh sweet orange fruits at ripening stages (Pan et al. 2012). In this study 48 proteins were identified by 2DE (two dimension electrophoresis) and MALDI-ToF-ToF mass spectrometry. These proteins were classified in groups (related directly and indirectly with carotenoid metabolism) such as heat shock proteins (e.g. HSP70, sHSP, chaperonin 60), anti-oxidative proteins (e.g. ascorbate peroxidase, cytosolic ascorbate peroxidase), stress response proteins (e.g. dehydrin, caspase), carbohydrate and energy metabolism (e.g. Rbcs, fructokinase-1, enolase), transcription and translation (e.g. RPL14, MYBC4), amino acid metabolism (e.g. glutamine synthetase, cysteine proteinase) and lipid metabolism (e.g. PAP). These results provided important information regarding which processes are related to carotenoid accumulation in sweet orange which could be further use for improvement of sweet orange (or other crops) fruit quality or lead to the discovery of new protein functions involved in these pathways.

One interesting gene/protein involved in carotenoid accumulation was discovered more than one decade ago. The *Orange* (*Or*) gene mutation in cauliflower (*Brassica oleracea var botrytis*) confers the accumulation of high levels of β-carotene in various tissues normally devoid of carotenoids (Lu et al. 2006). *Or* encodes a plastid-associated protein containing a DnaJ

Cys-rich domain, which is plant specific and highly conserved among divergent plant species. Its main role is associated to a cellular process that triggers the differentiation of proplastids (or other non-colored plastids) into chromoplast for carotenoid accumulation (Fig. 2). Manipulation of the *Or* gene led to increase of β-carotene content in different plant species (e.g. potato, sweet potato and Arabidopsis) making it a perfect target for biotechnological applications (Park et al. 2015; Zhou et al. 2015; Berman et al. 2017). Surprisingly, in potato tubers overexpressing the *Or* gene not only promoted accumulation of β-carotene but also a continuous stimulation of its accumulation during 5 months of cold storage (Li et al. 2012). An increase in carotenoid accumulation was found to be associated with the formation of lipoprotein-carotenoid sequestering structures and with the increased PSY abundance. Moreover, the provitamin A-carotenoids accumulated in the tubers were shown to be stable during simulated digestion and accessible for uptake by human intestinal absorptive cells (Li et al. 2012). This evidence suggest that *Or* transgene might be regulating or indirectly involved in other processes to facilitate the prolong accumulation of β-carotene. To unveil these processes, a comparative proteomic strategy using absolute quantitation (iTRAQ)-based shotgun proteomics was used. Due to the fact that cold storage also affects protein expression, authors compared protein expression between samples at 0 and 5 months of storage for the control and *Or* tuber samples. With this approach, a total number of 241 proteins (47 upregulated and 194 downregulated) was identified specifically in the *Or* tubers. Three major groups (chaperones and stress-responsive proteins, regulators of metabolic process and defense-response, and sugar metabolism and signalling) were identified among the upregulated proteins. This evidence suggests a very important role for chaperones, stress and metabolic regulators, and carbohydrate metabolism in the *Or*-regulated carotenoid accumulation (Li et al. 2012).

Similar to *Or*, another protein was discovered to be an enhancer of carotenoid accumulation in tomato (Kilambi et al. 2013). Tomato high-pigment mutants (e.g. DNA damage-binding protein1 (DDB1)/*hp1*, deetiolated1 (DET1)/*hp2*, zeaxanthin epoxidase (ZEP)/*hp3*, and intense pigment (unknown)/*ip*) exhibit enhanced carotenoid accumulation and chloroplast number. However, nothing was known about the mechanism underlying these phenomena. By using a comparative proteomic approach authors identified 72 proteins to be differentially expressed during ripening in *hp1* mutant in comparison to the wild type. Interestingly, *hp1* proteome showed an accumulation of proteins related with carotenoid sequestration and metabolism (e.g. chromoplast-specific carotenoid-associated protein/CHRC, violaxanthin de-epoxidase/VDE, ZEP). From those, CHRC showed increases at transcript and protein level in the *hp1* mutant compared to the wild type (but also in other mutants such as *hp2*, *hp3* and *ip*). Moreover, this protein was found together with carotenoids in plastoglobules, which are specialized structures for accumulation of carotenoids. Interestingly, based on previous proteomic studies in tomato, bell pepper and *Citrus* spp. (Siddique et al. 2006; Barsan et al. 2010; Zeng et al. 2011a), the CHRC protein was suggested to have a role in carotenoid storage. These evidences point CHRC as a key player in regulation of carotenoid sequestration and storage (Fig. 2), which seems to be very important for the enhanced accumulation of carotenoids in high-pigment tomato fruits.

In other important crop, the *PSY* and *CRTI* genes were overexpressed to obtain a golden rice variety (Datta and Datta 2006). To study the impact of carotenoid accumulation in the endosperm of this new variety proteomic and metabolomics analysis were performed (Gayen et al. 2016). The 2D-based proteomic analysis (MALDI-ToF MS/MS) showed two major groups with upregulated proteins by more than 2.5-fold. These groups correspond to carbohydrate metabolism (pullulanase, UDP-glucose pyrophosphorylase, glucose-1-phosphate adenylyl transferase, pyruvate phosphate dikinase/PPDK and phosphoglycerate kinase/PGK) and amino acid metabolism (prolyl hydroxylase). Increases in two proteins that catalyse consecutive reactions to produce pyruvate (precursor of the MEP pathway and carotenoids, amongst others) might explain the enhanced content of α-, β-carotene and lutein. This shed light into the importance of glycolytic process to support isoprenoid production, especially carotenoids.

All previous results are of remarkable value at the moment of deciding for a biotechnological application to enhance carotenoid production/accumulation in specific crop species.

6 Conclusions

Plastids are a very dynamic family of organelles that can differentiate into several specialized plastids (e.g. chloroplast, chromoplast, among others) depending on developmental or environmental cues. Proplastids are small undifferentiated plastids found in meristems and in reproductive tissues. In plants, all the specialized plastids are derived from proplastids that undergo a differentiation process (Fig. 2). The transition between green to red fruit in tomato represents the differentiation process in which chloroplasts differentiate to chromoplasts. This is a very complex process in which many molecular changes take place to coordinate the fruit ripening. With the advances made in the proteomic field, it was possible to characterize in detail the chloroplast to chromoplast transition during fruit ripening. The advantage of using proteomic analysis over other methods is the large amount of information that can be obtained from a single experiment. By using proteomics analysis it is possible to connect several processes (e.g. chlorophyll synthesis and degradation, carotenoid and lipid synthesis) that have to be coordinated during fruit ripening. First, in the (mature) green-stage of the tomato fruit there is still a predominance of photosynthesis-related proteins and chlorophylls. Moreover, proteins belonging to amino acid metabolism and protein synthesis were mainly expressed during cell division stage. In addition, during cell expansion proteins involved in photosynthesis and cell wall formation increased transiently to support the growth of the green fruit which is just starting (Faurobert et al. 2007). Between breaker and red stage of the tomato fruit there is a strong decrease in photosynthesis-related proteins as well as in chlorophyll content which is reflected in the absence of photosynthesis. Moreover, proteins involved in lipid metabolism and trafficking were very abundant, especially the ones involved in lipid-derived aroma volatiles (Barsan et al. 2010, 2012). In the red stage of the fruit the carotenoid content reaches its peak with a characteristic red color due to a great accumulation of lycopene.

Proteomic analysis also made a great contribution in the field of plastid proteolysis and carotenoid synthesis. Proteomic analysis revealed the composition and stoichiometry of the Clp protease, perhaps the most important stromal protease in the chloroplast (Olinares et al. 2011). This discovery allowed a better understanding of the function of this protease and led to the discovery of two substrate locators (e.g. ClpS and ClpF) (Nishimura et al. 2013; Nishimura et al. 2015). Further studies focusing in those subunits led to the identification of reliable Clp substrates (e.g. GluTR, pTAC17; Fig. 2) in Arabidopsis thaliana (Table 1). In the carotenoid biosynthetic pathway, proteomic analysis contributed with the reliable subcellular localization of the majority of MEP and carotenoid pathway enzymes (Joyard et al. 2009) in the chloroplast envelope, stroma and thylakoid membrane (Fig. 2). Furthermore, the discovery of the Or and CHRC proteins and its involvement in carotenoid accumulation and sequestration (Fig. 2; Table 1) opened new possibilities for genetic engineering of crops.

All the knowledge exposed here show how useful proteomic strategies are to obtain information not only about the protein of interest but also about protein interactors, their substrates, or processes directly/indirectly related with the function of the protein of interest (Li et al. 2012; Nishimura et al. 2013; Gayen et al. 2016). Moreover, proteomics can provide extensive information about the proteome in a specific developmental stage of the plant and explain which changes could have an impact in plastid or plant physiology (Barsan et al. 2010, 2012; Suzuki et al. 2015). It is important to mention that the value of proteomics can be enhanced by combination with other techniques (e.g. transcriptomics and metabolomics) or experimental designs (e.g. inducible promoters, affinity purification, etc.). Nevertheless, major analytical and technical advances in mass spectrometry provide the tools for fundamental discoveries and expansion of knowledge in plant science (e.g. plastid physiology) and other fields. Future efforts should aim for the designing of robust ways to analyse/interpret the data generated in proteomics studies (e.g. a good balance between mathematics and biology to define what is biologically significant) but also the use of integrative methods (e.g. transcriptomics and metabolomics) allowing researchers to build a wide picture of a particular process in the plant cell.

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