Running head: Proteome profile of hp1 tomato fruits

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Chromoplast specific carotenoid associated protein appears to be important for enhanced accumulation of carotenoids in *hpl* tomato fruits

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[W] The online version of this article contains Web-only data.
Abstract

Tomato (Solanum lycopersicum) high pigment mutants with lesions in diverse loci such as DDB1 (hp1), DET1 (hp2), zeaxanthin epoxidase (hp3) and Ip (Intense pigmentation, gene product unknown) exhibit increased accumulation of fruit carotenoids coupled with an increase in chloroplast number and size. However, little is known about the underlying mechanisms exaggerating the carotenoid accumulation and the chloroplast number in these mutants. A comparison of proteome profiles from the outer pericarp of hp1 mutant and the wild type fruits at different developmental stages revealed at least 72 differentially expressed proteins during ripening. Hierarchical clustering grouped these proteins into three clusters. We found an increased abundance of chromoplast specific carotenoid associated protein (CHRC) in hp1 fruits at red ripe stage which is also reflected in its transcript level. Western blotting using CHRC polyclonal antibody from bell pepper revealed a 2-fold increase in the abundance of CHRC protein in the red ripe stage of hp1 fruits compared with the wild type (WT; Ailsa Craig). CHRC levels in hp2 were found to be similar to that of hp1, whereas, hp3 and Ip showed intermediate level to that in hp1, hp2, and WT fruits. Both CHRC and carotenoids were present in the isolated plastoglobules. Overall, our results suggest that loss of function of DDB1, DET1, zeaxanthin epoxidase and Ip, up-regulates the CHRC levels. Increase in CHRC levels may contribute towards enhanced carotenoid content in these high pigment fruits by assisting in the sequestration and stabilization of carotenoids.
INTRODUCTION

Tomato high pigment mutants, \(hp1\) (\(hp1, hp1^w\)), \(hp2\) (\(hp2, hp2^j, hp2^dg\)), \(hp3\), \(Ip\) share not only characteristically high lycopene content, but also exhibit an increased number and size of chloroplasts in leaves, as well as in the green fruits. This leads to higher number of chromoplasts when the fruits ripen (Azari et al., 2010). \(HP1\) codes for DNA damage binding protein 1 (DDB1, Lieberman et al., 2004; Liu et al., 2004), while \(HP2\) encodes Arabidopsis homologue of DET1 (Mustilli et al., 1999) and \(hp3\) mutant has a lesion in zeaxanthin epoxidase (Galpaz et al., 2008). However, the gene product for \(Ip\) is still unknown (Lavi et al., 2009). Similar to \(hp1\) and \(hp2\) mutants, \(Ip\) also shows exaggerated photomorphogenesis (Lavi et al., 2009). Due to their high lycopene, flavonoid and vitamin contents, \(hp1\) and \(hp2\) mutations have been introgressed into various breeding populations. One such introgression from \(hp2^dg\) resulted in 3.5 fold increase in lycopene content in tomato fruits (Levin et al., 2003). Both DDB1 and DET1 seem to interact with Cullin4 and were found to be components of CUL4-based E3 ubiquitin ligase complex (Wang et al., 2008). CUL4-DDB1 complexes have been shown to affect overall plant development (Bernhardt et al., 2006) and flowering (Chen et al, 2010), where they may affect the epigenetic control of flowering. Double mutants of \(ddb1a\) and \(ddb1b\) showed that in Arabidopsis, DDB1 is critical for embryo development (Bernhardt et al., 2010).

Manipulation of light signaling components appears to be a good strategy to improve the tomato fruit quality as shown by fruit specific RNAi mediated suppression of \(DET1\) and repression of \(LeCOP1LIKE\) genes, which resulted in increased carotenoid levels (Davuluri et al., 2005; Liu et al., 2004). Similarly, repression of \(CUL4\) and fruit specific repression of \(HP1/DDB1\) by RNAi resulted in increased plastid compartment size and enhanced pigmentation of tomato fruits (Wang et al., 2008). Disrupting the function of all or any of these light signaling components seems to affect the plastid biogenesis, leading to an increased number of plastids with greater storage capacity for the carotenoids and/or pigments (Liu et al., 2004; Kolotilin et al., 2007). Likewise, the deficiency of ABA also seems to result in a similar high pigment phenotype (Galpaz et al., 2008). In all the above cases, the efficient conversion of chloroplasts to chromoplasts is necessary to accumulate the high amount of synthesized carotenoids.

A number of changes occur during the conversion of chloroplasts to chromoplasts. The first being the disintegration of thylakoid membranes, followed by loss of chlorophyll, an increase in the number of plastoglobules, accumulation of lycopene and an increase in the number of stromules, etc (Bian et al., 2011). Plastoglobules, besides accumulating lipids also
accumulate carotenoids either in the crystalline form as seen in tomato (Klee and Giovannoni, 2011) or fibrillar form as observed in bell pepper (Pozueto-Romero et al., 1997). In addition, these plastoglobules also appear to be involved in regulating carotenoid metabolism as enzymes such as ζ-carotene desaturase, lycopene β-cyclase and β-carotene hydroxylases are present in them (Ytterberg et al., 2006). Furthermore, proteins called plastoglobulins are associated with plastoglobules and help in accumulation of carotenoids. These include fibrillin (FIB), also known as CHRB or plastid lipid associated protein (PAP) from pepper chromoplast fibrils; chromoplast associated carotenoid binding protein (CHRC) and CHRD from petals of cucumber (Vishnevetsky et al., 1999). The expression of plastoglobulin genes appears to be under strict spatial and temporal regulation and well-coordinated with the expression of carotenoid biosynthetic genes like PSY1 in tomato fruits (Vishnevetsky et al., 1999; Giuliano et al., 1993).

All the plastoglobulins share a hydrophobic domain of 17-19 amino acids and this region seems to be important for carotenoid-protein interactions (Vishnevetsky et al., 1999). The significance of above mentioned plastoglobulins in the sequestration and the storage of carotenoids were elegantly exemplified in two studies (Simkin et al., 2007; Leitner-Dagan et al., 2006). Over-expression of a pepper fibrillin gene in tomato resulted in 2-fold increase in carotenoid levels as well as carotenoid derived volatiles (Simkin et al., 2007), where as down-regulation of CHRC in tomato led to a 30% reduction of carotenoids in the flowers (Leitner-Dagan et al., 2006). Moreover, a delayed loss of thylakoids was observed during chromoplastogenesis in fibrillin over-expressing tomato (Simkin et al., 2007). In addition, Or gene of cauliflower, which encodes a DnaJ cysteine-rich zinc finger domain containing protein, is also targeted to plastids and seems to control carotenoid accumulation by stimulating the formation of chromoplasts (Lu et al., 2006; Zhou et al., 2008; Li et al., 2012). All these studies suggest that manipulation of carotenoid sequestration and storage by stimulating chromoplastogenesis to create a metabolic sink might be an alternate strategy to increase carotenoid levels in food crops.

Currently, using systems biology approach, efforts are made to understand the complex interplay of ripening related pathways wherein transcriptome, proteome and metabolome are examined and attempts are made to interlink them (Osorio et al., 2011). Such a study in rin, nor and Nr mutants led to comprehensive understanding of ethylene regulated processes during ripening and also reinforced the need for integration of transcript, proteome and metabolite analyses (Osorio et al., 2011). In hpl mutant, transcription factor profiling
was combined with microarray and metabolite analyses and this study revealed that the secondary metabolism is controlled at the transcriptional level (Rohrmann et al., 2011). Using fruit-specifically down-regulated DET1 tomato lines, Enfissi et al. (2010) showed the significance of posttranscriptional regulation in modulating carotenoid and isoprenoid biosynthesis.

On the other hand, several proteomics studies have been carried out in tomato (Rocco et al., 2006; Faurobert et al., 2007; Manaa et al., 2011; Osorio et al., 2011; etc) as well as other fruits like strawberry (Bianco et al., 2009), grapeberry (Zhang et al., 2008), citrus (Zeng et al., 2011), etc and also in isolated chromoplasts (Barsan et al., 2010; Siddique et al., 2006). This lead to global understanding of the changes in protein profiles accompanying ripening. Several recent studies examining the linkage between gene expression and the metabolite levels during tomato fruit ripening, also highlighted the need for more comprehensive network approaches (Carrari and Fernie, 2006; Mounet et al., 2009; Rohrmann et al., 2011). Considering this, and the importance of hp1 mutant for improving the phytonutrient quality, we undertook a proteomic approach to decipher the role of DDB1 in tomato fruit ripening as information on proteome complement of hp1 is lacking. We compared the proteome profiles of outer pericarp in both WT and hp1 fruits at different stages during ripening. Though we did not find significant differences between WT and the mutant, we found a development-specific fine-tuning in the abundance of several proteins controlling multiple metabolic pathways in both WT and hp1 fruits, during ripening. We found higher levels of chromoplast associated carotenoid binding protein in hp1. Our results suggest a likely relationship between increased lycopene content and CHRC levels during fruit ripening.
RESULTS AND DISCUSSION

Influence of DDB1 on tomato proteome

High pigment \textit{1} fruits are characteristically different from the WT fruits in being more green in colour, at the MG stage (Fig. 1A) and accumulate more lycopene at the RR stage (Fig. 1A, B). In view of this, we characterized the proteome profiles at both these stages. We also included the BR stage, where the fruit makes transition, marking the onset of the ripening process. Consistent with the earlier reports, loss of function of \textit{DDB1} led to enhanced accumulation of lycopene in tomato fruits (Fig. 1B). In addition, the RR fruits of \textit{hp1} displayed 3-fold higher levels of lutein (Fig. 1C). Contrastingly, ethylene, an important regulator of fruit ripening, though elevated in \textit{hp1} at MG stage, declined considerably at the BR stage of fruit development (Fig. 1D).

A randomized block design was used to grow the tomato plants. The fruit sampling for all the three biological replicates was independently carried out as per the scheme shown in Supplemental Figure S1. For proteome profiling, we analyzed the proteins extracted from the outer pericarp of WT and \textit{hp1} fruits (Supplemental Fig. S2) at MG, BR and RR stages. The representative two-dimensional (2-DE) gel electrophoresis pictures comparing above three stages in WT and \textit{hp1} fruits are shown (Fig. 1E). Image analysis of the gels (n=3) with the Image master Platinum software revealed about 800 proteins in each gel. Seventy two proteins were found to be differentially expressed by at least 2-fold (over-expression or under-expression; statistically significant expression based on ANOVA with \(p \leq 0.05\)) in both mutant and wild type fruits, in at least one stage during ripening, representing about 9\% of spots on the gel. Notwithstanding the distinct phenotype of \textit{hp1} fruits, we did not find any proteins present specifically in WT or in mutant alone. This indicates that the subtle changes in proteome profile may be responsible for the specific phenotype of \textit{hp1} fruits. To ascertain this, we identified the molecular nature of differentially expressed proteins, to reveal any development specific fine-tuning in the expression of specific proteins in both mutant and WT. The differentially regulated protein spots (Fig. 1E, Fig. 2) were excised from gel, and after digestion with trypsin were subjected to MALDI-TOF analysis. Of the 72 spots, 51 spots could be successfully identified using Matrix Science’s Mascot search engine. For 17 spots, no firm identity could be assigned. For the remaining 4 spots, only a single peptide match was obtained that appeared to be statistically significant (Table S1). On comparing these differentially expressed proteins with the proteins identified from cherry tomato (Faurobert et al., 2007), tomato ecotypes (Rocco et al., 2006) and \textit{rin} mutant (Qin et al.,
our study revealed 14 additional proteins (Table S2), which were not detected in any of the above studies. Nevertheless, such variations between different studies are expected considering the diversity in the genotypes of different cultivars and mutants, sampling tissue and stages, the methods used for protein extraction, etc.

Functional distribution of the above identified proteins was performed according to the FunCat annotation and is shown in Supplemental Figure S3 (Ruepp at al, 2004). The differentially expressed proteins were distributed across 12 different categories, with nearly a half of the proteins being involved in carbon metabolism (46%) in accordance with massive up-regulation of metabolic processes such as respiration during fruit ripening. Among the remaining categories, stress response (14%) was maximally represented, followed by detoxification (7%), protein fate modification/degradation (6%), transport (6%), amino acid and hormone metabolism (4%), cell cycle regulation (3%), secondary metabolism (3%), replication (1%), protein synthesis and storage (1%), transcription (1%) and unclassified (8%).

Hierarchical clustering of identified proteins

To reveal patterns of protein expression, the above differentially expressed proteins were grouped. These proteins were clustered based on the unweighted pair group method with arithmetic mean (UPGMA) (Caraux and Pinloche, 2005) using the differences in protein spot intensity, at different ripening stages of both WT and hp1 fruits. The above analysis distinguished three main clusters of proteins, hereafter named as cluster I, II and III (Fig. 3A). As expected, in all the three clusters, proteins involved in carbohydrate metabolism were most abundant (Fig. 3B).

Cluster I (30.55% of all protein spots) included three different subclusters reflecting protein expression patterns. In the first subcluster, the expression patterns of proteins remained nearly uniform during the fruit development. This subcluster included 14 spots of which identity of six (37, 47, 10, 48, 3, 70) was not known. The remaining eight spots (52, 43, 8, 2, 11, 12, 13, and 42) belonged to proteins involved in carbohydrate metabolism. The second subcluster consisted of proteins whose level was similar at MG and RR stage but varied at BR stage. This cluster consisted of 5 proteins of which two were unidentified proteins (44, 34) and remaining three belonged to carbohydrate metabolism (17, 9, and 5). The third subcluster comprised of three proteins that declined during the fruit development (1, 6, and 7). While majority of the proteins belonged to housekeeping function, cluster I also included proteins regulating protein fate modification and degradation (11), detoxification (7), replication (52), and transport (1) (Fig. 3B).
Similar to cluster I, cluster II (33.33% of proteins) also included three subclusters. Subcluster 1 included proteins most of which were down-regulated at BR stage in WT (but with almost similar expression levels at MG and RR) but were up-regulated in hp1 and were specifically involved in carbohydrate metabolism (25, 23, 30, 29, 21, 45). The proteins which showed higher expression in the BR stage of hp1, with reduced but similar expression levels at MG and RR, were classified in subcluster 2 (36, 38, 28, 51, 15, 19, 22 and 50). While most of these proteins were part of photosynthesis machinery, this also included a 17.7 sHsp (small heat shock protein, 50). Subcluster 3 consisted of proteins that showed almost constant expression levels in both WT and hp1 (24, 33, 18, 16, 20, 32 and 63) during ripening. These proteins belonged to carbon metabolism (24, 32), amino acid/hormone metabolism (18, 33), cell cycle regulation (16), secondary metabolism (20), transport (40) and detoxification (63) (Fig. 3B).

Cluster III (36.12% of proteins) comprised of proteins with diverse biological functions showing similar expression patterns in either WT or hp1 across different developmental stages during ripening. In WT fruits, this cluster included proteins modulating stress (60, 68, 62, and 65), detoxification (49), hormone metabolism (41), secondary metabolism (58) and rest of unknown function (72, 71, 57, and 54) (Fig. 3B). The spots that were specifically modulated in hp1 fruit consisted of proteins involved in the protein fate modification/degradation (56, 46); detoxification (49), stress response (61) and few are of unknown function (39, 57 and 71) (Fig. 3B). One of the spots (66) showed a diametrically different expression pattern in WT and hp1. It had higher expression in WT at MG stage and declined at subsequent BR and RR stages, while in hp1, its expression progressively increased from MG to RR stages (Fig. 3A).

The metabolic assignment of variably expressed proteins during the fruit ripening revealed three main pathways. First pathway comprised of the proteins that are involved in the Calvin-Benson cycle, Glycolysis, TCA cycle and the interconnecting pathways (Supplemental Fig. S4). The reactions related to photosynthetic electron transport in the plastids constituted the second pathway (Supplemental Fig. S5). The third pathway (Supplemental Fig. S6) is a model showing the probable mode of action of differentially expressed heat shock proteins, detected in our study.

*HP1* shows accumulation of proteins related to carotenoid sequestration and metabolism

High pigmentation of fruits in tomato appears to be regulated by diverse set of genes encoded by distinct genetic loci, *hp1, hp2, hp3* and *Ip* (Azari et al., 2010). At RR stage, fruits
of all these mutants possess elevated level of lycopene and β-carotene, signifying commonality in up-regulation of these carotenoids. In view of this, we specifically examined among the 72 differentially abundant proteins for the presence of specific enzymes or proteins that may have assisted in carotenoid accumulation either by regulating biosynthesis, sequestration or degradation. We found three such proteins. Lipocalins are lipid associated transporter molecules, helping in transport of certain lipids and small molecules (Frennette et al., 2002). They also include enzymes like violaxanthin deepoxidase and zeaxanthin epoxidase (Hieber et al., 2000), which play a role in carotenoid degradation. During ripening the abundance of temperature induced lipocalins increased in both WT and hp1, although the expression at BR stage was less in WT and thereafter increased at RR stage. Similar increase in levels of lipocalins was also observed in other tomato proteomics studies (Faurobert et al., 2007; Osorio et al., 2011). It is known that alcohol dehydrogenase (ADH) is an important enzyme in the biosynthesis of volatiles in tomato (Speirs et al., 1998). We found ADH to be up-regulated in both WT and mutant at RR stage, but the protein level was much higher in WT. The observed increase in ADH and lipocalins at RR stage may be related to increase in the levels of compounds contributing to volatile/flavor formation in tomato fruits.

One protein that showed an interesting expression pattern is the chromoplast associated carotenoid binding protein (CHRC). This protein showed a gradual decline in abundance during ripening in WT. On the other hand, in hp1, the protein showed an opposite trend; it progressively increased, attaining a peak at RR (Fig. 3). This protein was also found in the chromoplast proteome studies of tomato, bell pepper, and citrus (Barsan et al., 2010; Siddique et al., 2006; Zeng et al., 2011), and it is assumed that it plays a role in carotenoid storage.

Expression profiling of genes involved in carotenoid precursor and carotenoid biosynthesis

Though it is logically expected that high carotenoid levels in hp1 fruits should reflect in up-regulation of enzymes of the pathway, our proteome analysis did not reveal any differentially expressed protein spots for any of the carotenoid biosynthetic enzymes. Apparently, the levels of these enzymes in both WT and hp1 were below the detection limits of colloidal coomassie blue staining and it is believed that the flux in the carotenoid biosynthetic pathway is mainly modulated by posttranscriptional, translational or posttranslational regulation. To ascertain the influence of hp1 mutation on transcript level, we examined the expression of genes encoding the entire carotenoid biosynthetic pathway.
Figure 4 shows the expression profiles of 20 genes using quantitative real time PCR analysis involved in carotenoid synthesis, including genes contributing to the formation of first precursor phytoene. Despite the accumulation of higher levels of lycopene in \textit{hp1} fruits, the transcripts of genes belonging to the carotenoid pathway regulating the formation of phytoene and its desaturation to lycopene, did not show any concerted up-regulation at BR and RR stages when compared to WT. The absolute levels of different transcripts varied distinctly and could be classified into two groups. In WT fruit at MG stage, \textit{LCYB1}, \textit{LCYB2}, \textit{ZEP} and \textit{NCED1} were the most abundant transcripts (>1.0-8.0), whereas other genes were expressed at lower level (0.0-1.0). A comparison of WT gene expression with \textit{hp1} fruit at MG stage indicated that the expression of most genes was significantly down-regulated in the mutant, except \textit{CYP97A29}, \textit{CYP97C11}, \textit{CRTRB1} and \textit{CRTRB2}, which were up-regulated.

In WT \textit{GGDS2}, \textit{LCYB2} were the most abundant transcripts (>1.0-13.0) at BR stage whereas other genes were expressed at lower level. While the expression levels of \textit{DXS}, \textit{IDI}, \textit{GGDS2}, \textit{PSY1}, \textit{ZDS}, and \textit{VDE} were nearly similar, other transcripts such as \textit{PSY2}, \textit{ZIS}, \textit{CRTISO}, \textit{LCYB2}, \textit{CYCB}, \textit{LCYE}, and \textit{CRTRB2} were down-regulated in \textit{hp1} compared to WT. At BR stage, the level of \textit{PDS}, \textit{ZEP}, \textit{CYP97A29}, \textit{CYP97C11} and \textit{CRTRB1} transcripts were significantly higher in \textit{hp1} mutant than WT.

At RR stage \textit{DXS} and \textit{ZEP} were the most abundant transcripts (>1.0-24.0) in WT, whereas other genes were expressed at lower level. In RR fruits, transcript levels of \textit{DXS}, \textit{PSY2}, \textit{LCYB1}, \textit{LCYE}, and \textit{NCED1} were nearly similar in WT and \textit{hp1}, whereas, \textit{IDI}, \textit{GGDS2}, \textit{PSY1}, \textit{ZIS}, \textit{CRTISO}, \textit{LCYB2}, \textit{CRTRB2}, \textit{ZEP} were down-regulated in \textit{hp1}. In contrast, expression levels of \textit{PDS}, \textit{ZDS}, \textit{CYCB}, \textit{VDE}, \textit{CYP97A29}, \textit{CYP97C11} and \textit{CRTRB1} were higher in \textit{hp1} mutant. Among these seven transcripts, the gene products of only \textit{PDS} and \textit{ZDS} contribute to lycopene, whose levels are elevated in \textit{hp1} mutant. The slight stimulation of β-carotene level in \textit{hp1} fruits may perhaps be related to up-regulation of \textit{CYCB} transcript at RR stage. Similarly, the gene products of \textit{CYP97A29}, \textit{CYP97C11}, \textit{CRTRB1} seems to contribute to higher lutein levels found in the RR fruits of \textit{hp1} as their transcripts are up-regulated. Our results are in agreement with the study of Stigliani et al., (2011) reporting higher expression of \textit{CYP97A29}, \textit{CYP97C11} and \textit{CRTRB1} during tomato ripening.

Of interest was the opposite expression patterns of \textit{ZEP} and \textit{VDE} transcripts, with down-regulation of \textit{ZEP} and up-regulation of \textit{VDE} in \textit{hp1} mutant. While gene product of \textit{ZEP} converts zeaxanthin to violaxanthin, the gene product of \textit{VDE} reconverts violaxanthin to zeaxanthin. The reduced expression of \textit{ZEP} and enhanced expression of \textit{VDE} in \textit{hp1} appears to be in agreement with the characteristic increase in chloroplast number and size which
could be correlated to reduced ABA levels in hp1 (Carvalho et al., 2011). A similar phenotype was also observed in ABA deficient hp3 mutant, which has a lesion in ZEP (Galpaz et al., 2008). A link between HP1 transcript levels with ABA level is also apparent from observations that the application of external ABA stimulates expression of DDB1 mRNA in tomato seedlings (Wang et al., 2008).

Despite the distinctive increase in lycopene level in hp1 fruits, the transcript levels of most enzymes of carotenoid biosynthesis pathway were lower than that of WT at all stages of fruit ripening. The predominant down-regulation of transcripts in hp1 barring few such as PDS, ZDS, CYCB and VDE that show up-regulation at RR stage, indicates that enhanced carotenoid formation in hp1 mutant is most likely regulated at post-transcriptional level. Similar to this study, Enfissi et al. (2010) also suggested the post-transcriptional regulation of carotenoid biosynthesis pathway, as they did not find correlation between transcript profiles of carotenoid biosynthetic genes and changes in the carotenoid levels in DET1 down-regulated tomato fruits. One of the enzymes that may regulate flux into carotenoid biosynthesis pathway is phytoene synthase I. It is believed that increase in phytoene level has a feed forward effect on carotenoid biosynthesis (Fraser et al., 2007). It is also reported that activity of PSY1 enzyme in hp1 was up-regulated by 2-fold though there was no corresponding increase in the transcript of PSY1 and PDS (Cookson et al., 2003), suggesting a regulation of PSY1 activity at the post-transcriptional level.

A system biology approach is required to uncover hp1 contribution to fruit ripening

The emergence of “omics” have highlighted the need for systems biology approach as a tool for deciphering links between coexpressed genes and pathways and identifying master regulatory points governing a biological response (Saito et al., 2007). The expression of a metabolome phenotype during development of an organism results from a complex interaction between multiple responses at several levels right from the expression of genes regulating the pathway till the final biosynthesis of metabolites. In order to understand the complexities of regulation of metabolic networks during fruit development in tomato, it is essential to correlate data from protein, transcript and metabolite experiments. In view of this, we attempted to correlate the proteome data obtained in this study with the transcript data available at www.ted.bti.cornell.edu and metabolite data from Rohrmann et al. (2011). Out of 72 differentially expressed proteins that varied during fruit development, transcripts for only 18 proteins were available on the TOM1 microarray. Furthermore, a detailed examination of the gene expression data revealed that in TOM1 microarray, the transcript profiles obtained at 7 dpa of fruit development were used for normalizing all other data at respective stages (7
dpa - 57 dpa). However, we obtained proteome data only at three different ripening stages, MG, BR and RR. Since we did not include a time point corresponding to 7 dpa in our study, a direct correlation to this data set was ruled out.

As an alternative, we compared the transcript profiles for five carotenoid pathway genes obtained in the current study with the corresponding metabolite data obtained earlier in WT and hp1 at RR stage (Cookson et al., 2003). These transcript-metabolite pairs include PSY1-phytoene, ZIS-ζ-carotene, ZDS-lycopene, CRTISO-lycopene and CYCB-β-carotene. The above pairs were correlated using Pearson correlation algorithm with XLSTAT 2012 software (Supplemental Figure S7A). The correlation coefficient (R) values indicated a negative correlation between transcripts and metabolites in WT. Despite the high expression of PSY1 and CRTISO in WT, low lycopene levels were observed at RR stage. On the contrary, a poor correlation was observed in the mutant, where ZDS was up-regulated but CRTISO was down-regulated yet resulted in higher lycopene levels in hp1 fruits. Similar to our study, a lack of correlation between transcript and metabolite profiles leading to lycopene formation was also observed in the case of DET1 down-regulated tomato lines (Enfissi et al., 2010) and also in PSY1 over-expressing tomato lines (Fraser et al., 2007). At the same time in DET1 down-regulated tomato lines (Enfissi et al., 2010) a strong correlation between ripening-associated transcripts and specific metabolites, such as organic acids, sugars, and cell wall-related metabolites was observed underlining the diversity in regulation of metabolic networks in fruit ripening. The negative correlation between carotenoid pathway transcripts and carotenoids indicates the importance of post-transcriptional regulation in elevating carotenoid levels in hp1 fruits. This view is supported by the observation that though there was no corresponding increase in the transcript of PSY1 and PDS, the activity of PSY1 enzyme in hp1 was up-regulated by 2-fold (Cookson et al., 2003). Similar lack of correlation was also observed between transcript profiles of CYP97A29, CYP97C11, CRTRB1, and CRTRB2 and the lutein levels in WT and hp1 fruits (Supplemental Figure S7B). Though the R values obtained were higher in hp1 compared to WT, they do not indicate a strong correlation between the transcripts and the lutein levels. The absence of correlation between transcript levels and respective metabolites (lutein and lycopene) indicate that the alternate mechanisms like post-transcriptional regulation might be controlling the carotenoid pathway.

Similarly, we correlated the protein (for 5 differentially expressed proteins) and the corresponding metabolite data across different developmental stages in both WT and hp1 (Rohrmann et al., 2011). Acid invertase (spot 53)-glucose, UDP glucose pyrophosphorylase
(spot 17)-sucrose, SAM synthetase (spot 18)-ethylene, E8 protein (spot 41)-ethylene and Malate dehydrogenase (spot no. 42)-malate protein-metabolite pairs were analyzed by XLSTAT (Supplemental Fig. S7C, D). The resulting R value suggested a negative correlation between proteins and metabolites at MG and BR stages for both WT and mutant. On the contrary, at RR stage, the R values were very less, indicating weak or no correlation between proteins and metabolites in both WT and hp1. On the whole our results highlight the need for a comprehensive systems approach including post-translational regulation, feedback regulation, metabolite turnover, and partitioning between different organelles and/or locations in the cell, to uncover linkage between metabolic networks regulating fruit ripening (Fernie and Stitt, 2012).

**Loss of function of DDB1 enhances the abundance of CHRC**

A comparison of the protein profiles in WT and hp1 fruits revealed an increase in the abundance of CHRC (spot 66) at RR stage of hp1 whereas its level declined in WT at the same stage. A detailed chronological analysis revealed an interesting pattern of abundance of CHRC, with WT and hp1 following dramatically opposite patterns. Based on the intensity of spot volumes, in WT, CHRC level was highest at MG, followed by a decline to nearly similar levels in both BR and RR. In contrast, in hp1, CHRC level increased from MG to RR stage, with maximal level at RR stage (Fig. 5A). Analysis of CHRC transcript levels (Fig. 5B) corroborated the above pattern of CHRC protein levels, albeit with few differences. In MG stage, WT exhibited slightly higher CHRC expression (1.7 fold) than hp1. At BR stage, WT showed 3-fold increase in CHRC expression than hp1, but at RR stage, hp1 showed around 6.35-fold higher expression than WT. hp1 showed highest CHRC expression in BR, followed by RR stages (Fig. 5B). But the protein profile revealed the opposite, with high abundance at RR stage, suggesting post-transcriptional regulation of CHRC.

CHRC was first discovered in cucumber corollas (Smirra et al., 1993) and was found to be a lipid binding protein. Survey of literature revealed that this protein was extensively studied in bell pepper (Deruere et al., 1994) and was named as plastid lipid associated protein (PAP) and antibodies against this protein were also raised. Comparison of protein sequences of bell pepper PAP and tomato CHRC revealed 89% homology (Supplemental Fig. S8A, C). Also, tomato CHRC protein showed 100% sequence similarity to tomato PAP protein sequence, suggesting that these two proteins are similar (Supplemental Fig. S8A). On the contrary, alignment of tomato CHRC/PAP with tomato fibrillin family members (10 members based on blast search in SGN database, Supplemental Fig. S8B) revealed high degree of mismatches except for two sequences (Solyc08g005220.2.1; Solyc08g076480.2.1),
where homology of 41 and 50% respectively was found (Supplemental Fig. S8B). The divergence in CHRC sequence with fibrillin indicated that while these two proteins are different, the occurrence of PAP domains in fibrillin protein sequences led to their classification as PAP family proteins. Nevertheless, both CHRC and fibrillin appear to play a major role in storage of carotenoids in the chromoplasts (Pozueta-Romero et al., 1997; Vainstein et al., 1994).

Given the strong homology between bell pepper PAP and tomato CHRC, bell pepper PAP antibody recognized a tomato protein of molecular weight 35±2 kD at a location identical to bell pepper PAP, albeit the level of this protein was quite high in bell pepper than in tomato (Fig. 5C). We considered the possibility that whether bell pepper antibody recognized any protein other than CHRC. A comparison of molecular weights of fibrillin proteins showed that among all fibrillins, only one fibrillin (hit 7, accession no. Solyc08g076480.2.1) had a molecular weight of 37.48, which was nearly similar to that of tomato CHRC (Supplemental Fig. S8C). However, this accession showed only 50% homology to tomato CHRC (Supplemental Fig. S8B). Therefore, the probability of this protein being recognized by bell pepper antibody is minimal as alignment of peptides obtained after MALDI analysis of tryptic peptides from spot 66, showed 100% alignment with CHRC (Supplemental Fig. S8D) but only little homology with fibrillin hit 7 (Supplemental Fig. S8E). The proteome analysis also did not show any other differentially expressed spot belonging to fibrillin hit 7. Therefore, in all probability, the bell pepper antibody recognized tomato CHRC protein on immunoblots. Moreover the observed increase in level of CHRC during ripening is also supported by an increase in its transcript level (Fig. 5A, B).

A comparison of CHRC protein levels in both WT and hp1 fruits at RR stage revealed at least 2-fold higher CHRC level in hp1 than in WT (Fig. 5D). At the MG stage, while CHRC band was discernible in hp1, in WT it was below the detection limits. Immunoblotting indicated that hp1 fruits show higher level of CHRC protein and its level increased during the ripening (Fig. 5E). We considered the possibility that an increase in lycopene level in hp1 may have stimulated an increase in the CHRC level in hp1 fruits. To ascertain this, we examined the CHRC levels in nor mutant that accumulates little lycopene and in Arka vikas (AV), an Indian cultivar which has lycopene level similar to Ailsa Craig (AC; the progenitor of hp1 mutant). Despite the low level of lycopene in nor, it accumulated CHRC protein at a
level almost equal to the other two cultivars AV and AC (Fig. 5F), thus ruling out the possibility that an increase in lycopene level may have stimulated the CHRC increase.

In another study, Lenucci et al. (2012) observed the enhanced level of a 41.6 kD protein (unidentified) in chromoplasts purified from mesocarp of high lycopene accumulating cultivar of tomato, HLY-18 in comparison with a traditional cultivar. However, the protein profiles of hp1 fruits did not reveal any specific increase in 41.6 kD protein. The observed variation between our study and that of Lenucci et al. (2012) could also be due to cultivar differences or differences in the tissue used as we did protein profiling from outer pericarp only.

Since mutation in DET1 (hp2, Mustilli et al., 1999) also leads to high pigment phenotype, it was of interest to examine whether this mutant too exhibits high levels of CHRC protein. Analysis of CHRC levels from hp2 mutant fruits at both MG (Fig. 5G) and RR stages (Fig. 5H) revealed an abundance level similar to hp1, suggesting an overlap in the action of hp1 and hp2 loci in regulating CHRC levels. Though DDB1 (hp1) and DET1 (hp2) are diverse loci, the observed overlap may have emanated from their coaction as part of CUL4-DDB1-DET1 complex (Wang et al., 2008), where loss of function of either of these proteins may be contributing to higher CHRC and also pigment levels. Consistent with enhanced expression of CHRC protein in hp2, Enfissi et al. (2010) showed increase in the number and size of plastoglobules in red ripe fruits of DET1 down-regulated lines. Similarly, it was reported earlier that chloroplast to chromoplast transition in tomato is accompanied by an increase in number and size of plastoglobules (Harris and Spurr, 1969). Taken together the above observations, the increased levels of CHRC protein in both hp1 and hp2 fruits may be linked to an increase in the abundance of plastoglobules where higher CHRC levels in chromoplasts assist in sequestration and stabilization of carotenoids, thus boosting their level.

The specific role of CHRC in chromoplasts is also supported by the observation where down-regulation of tomato CHRC using RNAi strategy led to 30% reduction of total carotenoids in tomato flowers (Leitner-Dagan et al., 2006). Since RNAi suppression of CHRC reduced carotenoid level, the over-expression should increase its level. Though there are no reports on CHRC over-expression in literature, over-expression of related protein, fibrillin from pepper which shares PAP domain with CHRC in tomato, resulted in almost doubling of carotenoid levels and carotenoid-derived aroma compounds in tomato fruits (Simkin et al., 2007). Evidences are emerging for additional roles for plastoglobulin proteins like CHRC, which not only participate in sequestration of carotenoids, but also appear to influence chromoplastogenesis as well as carotenoid metabolism (Brehelin and Kessler,
2008; Li et al., 2012). Taken together, up-regulation of CHRC in chromoplasts supports the role of this protein in chromoplast-specific carotenoid storage (Leitner-Dagan et al., 2006).

Similar to hp1 and hp2 mutants, Intense pigmentation (Ip) mutant exhibits high lycopene content in fruits and also has similar increase in the number and size of chloroplasts but the gene responsible for this phenotype is not yet identified (Jones, 2000; Lavi et al., 2009). Kendrick et al. (1997) reported that Ip displayed photomorphogenic responses intermediate between hp1 and normal genotypes. In view of its high pigment phenotype, we examined whether Ip also exhibits high level of CHRC protein in ripened fruits. Western analysis revealed that similar to hp1 and hp2, Ip also up-regulates CHRC protein but has levels intermediate to that in WT and hp1, hp2 fruits (Fig. 5H). Consistent with the intermediate CHRC level, the red ripe fruits of Ip mutant also showed intermediate level of lycopene and β-carotene to that of WT and hp1 mutant (Jones, 2000). This suggests that increase in chloroplast number, size and lycopene level in Ip is likely regulated by a mechanism similar to that in high pigment mutants and thus shows intermediate increase in CHRC levels.

Since a deficiency in ABA could also lead to a high pigment phenotype (hp3), it was of interest to examine the CHRC levels in this mutant. Monitoring of CHRC levels by Western blotting in the RR fruits of hp3 mutant revealed a lower abundance of CHRC compared to that in hp1, nonetheless, it was higher than that of the WT (Fig. 5I). This result is corroborated by the observation that the deficiency of ABA in hp3 is accompanied by an increase in plastid compartment size due to increased plastid division (Galpaz et al., 2008). This could result in a higher storage capacity for the carotenoids similar to hp1 and hp2 mutants. Though hp3 is not a member of CUL4-DDB1-DET1 complex (Wang et al., 2008), this mutation may affect the carotenoid levels through feedback regulation (Galpaz et al., 2008).

In view of the observed differences in the CHRC abundance in the hp1, hp2, hp3 and Ip, it was of interest to examine the transcript levels of CHRC in the RR fruits of all these four mutants (Fig. 5J). In comparison to WT, hp1 showed highest expression of CHRC (6.35-fold), followed by hp2 (4.12-fold), Ip (2.37-fold) and hp3 (1.37-fold) (Fig. 5J). These transcript profiles appear to be consistent with the protein profiles determined by Western blotting, except in the case of hp2. Nevertheless, all the four mutants exhibited high transcript as well as protein levels for CHRC compared to WT, which is consistent with their high
pigment phenotype, where this protein might have a major role in sequestering the elevated carotenoids.

Both carotenoids and CHRC are present in plastoglobules

The transition from chloroplasts to chromoplasts during ripening is accompanied by an increase in osmiophilic bodies associated with the thylakoids named as plastoglobules. The chromoplasts of hp1 mutant show 1.3-fold increase in size compared to wild type (Cookson et al., 2003). Likewise down-regulation of DET1 leads to an increase in the plastoglobule number in the chromoplasts (Enfissi et al., 2010). Biochemical analysis of plastoglobules revealed that these are enriched in lipids and also contain several proteins including enzymes participating in carotenoid metabolism (Brehelin and Kessler, 2008, Austin et al., 2006). Based on the enrichment of lipids in the plastoglobules it is assumed that carotenoids are also present in them. Since, CHRC has been implicated as a protein that assists in the sequestration and storage of carotenoids, and it may be presumably localized in plastoglobules, we examined the presence of carotenoids and CHRC in the isolated plastoglobules. We purified the plastoglobules from the RR fruits of tomato by taking advantage of their lower density compared to other membranous fractions on sucrose density gradients (Fig. 6A) by adapting the procedures of Besagni et al., (2011) and Lundquist et al., (2012). The purity of plastoglobule fraction and fluorescence emission from globuli were examined by confocal microscopy (Fig. 6B-D), which revealed the presence of intact globules (Fig. 6D). Moreover, the purified plastoglobules exhibited a bright green autofluorescence on exposure to 488 nm argon laser, indicating the enrichment of carotenoids (emission in the range of 500-510 nm; Fig. 6B, E), whereas they exhibited only a trace level of red autofluorescence (740-750 nm, Fig. 6C), indicating a near absence of chlorophylls. Since the plastoglobules were isolated from the red ripe fruits, absence of chlorophyll was expected akin to an earlier observation for the isolated chromoplasts (Egea et al., 2011). In addition, carotenoids were extracted from the plastoglobule fraction and the absorbance spectrum of the extract showed a distinct peak in the 440-475 nm range which is characteristic of carotenoids (Fig. 6F). From the foregoing observations it is evident that the plastoglobules isolated from RR fruits are enriched in carotenoids.

To ascertain the presence of CHRC in the purified plastoglobules, the proteins were extracted from the plastoglobule fraction and were resolved by SDS-PAGE, followed by Western blotting. Figure 6G shows the presence of CHRC protein in purified plastoglobules. The comparison of CHRC levels in plastoglobules with other fractions indicated a substantial
enrichment of the CHRC protein in the plastoglobules, than in the chromoplast membrane fraction, or in the initial fruit extract. The presence of CHRC in the chromoplast membrane fraction (prior to its sonication to release the plastoglobules) is also in conformity with an earlier transmission electron microscopic observation that the plastoglobules are associated with chromoplast membranes in the ripe fruits (Harris and Spurr, 1969).

The up-regulation of CHRC in the fruits of four diverse mutants sharing a high carotenoid phenotype suggests an interrelationship between carotenoid accumulation and the CHRC levels. Several studies have indicated that the CHRC and related proteins; fibrillins enhance the sequestration of carotenoids into plastoglobules (Brehelin and Kessler, 2008). The importance of carotenoid sequestration and storage is highlighted in studies where attempts to enhance the carotenoid levels were mitigated by the absence of a proper sink. In potato tubers a massive loss of carotenoids occurs during long term storage due to enzymatic or non-enzymatic oxidation of carotenoids. However, such loss can be prevented by expression of cauliflower Or gene, which stimulates the formation of plastoglobule-like structures storing carotenoids in potato tubers. The sequestration of the carotenoids in the above structures prevents oxidation of carotenoids and ensures continued formation of carotenoids during storage of tubers (Li et al., 2012). It is likely that higher CHRC levels function to abet the storage of carotenoids and protect them from degradation in high pigment mutants. However, it remains to be investigated whether up-regulation of CHRC by genetic or transgenic means would improve the storage capacity of carotenoids in tomato fruits.

In summary, our results suggest that the loss of function of DDB1, DET1, zeaxanthin epoxidase and Ip in tomato fruits appears to up-regulate CHRC protein levels in a manner that is currently unknown. More studies are needed to determine how these diverse alleles regulate CHRC levels in tomato fruits.

Conclusions

In this study using a proteomic approach we examined the influence of DDB1 on tomato fruit ripening. At least 72 proteins were differentially expressed in both WT and hp1 whose expression was fine-tuned in response to developmental cues during fruit ripening. We found an additional role for DDB1 and DET1 in up-regulating the level of chromoplast associated carotenoid binding protein by 2-folds by an unidentified mechanism. hp3 mutant, which is deficient in zeaxanthin epoxidase also exhibits higher CHRC level, albeit at a lower level compared to hp1 and hp2 mutants. This is consistent with its enlarged plastid compartment size and elevated carotenoid content. The intermediate level of CHRC in Ip
appears to be related to its intermediate photomorphogenic phenotype and carotenoid levels between WT and hp1, hp2 mutants. Interestingly, transcript profiling of carotenoid pathway genes revealed an overall up-regulation of the pathway in WT rather than in hp1, except the desaturation-steps catalysed by PDS and ZDS, suggesting a post-transcriptional control of the pathway. Moreover, none of the carotenoid biosynthetic enzymes were detected in our proteomics studies either because their levels were below detection limits or they were regulated at translation level. However, the transcript levels of cytochrome P450 carotenoid β- and ε-hydroxylases responsible for the conversion of α-carotene to lutein were up-regulated in the hp1 mutant than in WT, consistent with the elevated lutein levels found in the mutant. Compared to WT, transcript levels for ABA biosynthetic genes were down-regulated in hp1, suggesting a relation between low ABA levels and increase in chloroplast number and size as previously reported in hp3 mutant. A poor correlation was observed between transcript, protein and metabolite levels reinforcing the need for systems biology approaches for complete understanding of the metabolite networks. In summary, our study revealed that CHRC likely regulates carotenoid sequestration and storage, which seems to be important for enhanced accumulation of carotenoids in high pigment tomato fruits.
MATERIALS AND METHODS

Plant material and experimental design

Fruits of tomato photomorphogenic mutant, high pigment1 (hp1) and its isogenic wild type (WT) Ailsa Craig (AC, Srinivas et al., 2004) were used for proteomic analysis in this study. The tomato accessions AC (LA 2838), hp1 (LA 3538), hp2dx (LA 2451), Ip (LA 1500), hp3 (3-343) and nor (LA 3770) were obtained from tomato genetics resource centre (TGRC, University of California, Davis). Tomato plants were grown under natural photoperiod in the greenhouse at 28±1°C during day and ambient temperature in night. Plants of both WT and hp1 were grown in three randomized blocks (each block constitutes one biological replicate), with each block consisting of 3 rows, with 5 plants each for WT and hp1/row (Supplemental Fig. S1). To minimize variation, fruits developed only on the first and second truss were used for experiments. The flowers were tagged at the time of anthesis for chronological monitoring of fruit developmental stages and different ripening stages-mature green [MG, 35 days post anthesis (dpa)], breaker (BR, 40 dpa) and red ripe (RR, 49 dpa) stages were identified (Fig. 1A). From each block (biological replicate), 30-35 fruits/ripening stage/genotype were harvested and the outer pericarp was immediately isolated as described below, pooled separately at every ripening stage for both WT and hp1 (Supplemental Fig. S1) and flash frozen in liquid nitrogen and stored at -80°C until use.

Plant material for protein, transcript and carotenoid analyses

Fruits of WT and hp1 were collected at different ripening stages as described above. Since a temporal difference in the synthesis of carotenoids in the outer pericarp and inner pericarp was observed in hp1 mutant (unpublished results), we initiated an independent study on both outer and inner pericarp. Outer pericarp is the outer layer of the fruit including the skin as indicated in the Fig. S2 (Supplemental Fig. S2); its thickness is measured with a vernier calipers and it varies from 0.5±0.1 mm in MG/BR stage to 0.3±0.1 mm in RR stage, (Supplemental Fig. S2). From fruits at different ripening stages and from all biological replicates, outer pericarp was carefully excised using a scalpel, pooled, flash frozen in liquid nitrogen and stored at -80 till further use. Unless specifically indicated, this tissue was used for proteome, transcript and carotenoid analyses.

Protein extraction

Proteins were extracted from the outer pericarp of fruit tissue using a previously described protocol (Isaacson et al., 2006) with slight modifications. About 1 g of tissue was homogenized in liquid nitrogen and suspended in 7 mL of extraction buffer containing 0.7 M
sucrose, 0.1 M KCl, 0.5 M Tris pH 7.5, 50 mM EDTA, 50 mM DTT, 1 mM PMSF and 25 µL protease inhibitor cocktail (Sigma-Aldrich, St. Louis, USA). To this an equal volume of Tris-saturated phenol was added and the sample was mixed thoroughly by shaking at 4ºC for 30 min. The mixture was centrifuged at 20,000g for 30 min at 4ºC. The upper phenolic phase was collected and re-extracted 2 more times as described above. The protein in the phenolic phase was precipitated at -80°C by adding 5 volumes of 0.1 M ammonium acetate containing 50 mM DTT and protein was pelleted by centrifugation at 26,200g for 30 min at 4°C. The protein pellet was washed twice with methanol containing 10 mM DTT and once with acetone with 10 mM DTT, and finally dissolved in lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) 3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate (CHAPS)]. The protein was quantified using Amido Black method (Goldring and Ravaiolli, 1996). For each developmental stage, at least three biological replicates were used in three independent extractions.

Two-dimensional Gel Electrophoresis (2-DE)

Proteins (500 µg) were dissolved in 250 µL rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 50 mM DTT and 1% (v/v) ampholytes (4-7 pH)] and applied on to 13 cm immobiline dry strips, pH 4-7 (GE Healthcare) by passive rehydration overnight. IEF was carried out at 20°C in Ettan IPGPhor 3 (GE healthcare) and the settings used were as follows: 150 V for 1 h, 250 V for 1 h, 500 V for 1 h, 1000 V for 30 min, 8000 V for a total of 40,000 Vh with a maximum current of 50 µA/strip. After isoelectric focusing (IEF), the strips were equilibrated first in buffer containing 6 M urea, 0.075 M Tris, 2% (w/v) SDS, 30% (v/v) glycerol and 2% (w/v) DTT for 20 minutes. In the second step, 2.5% (w/v) Iodoacetamide was used instead of DTT in the same buffer for another 20 min. Then the strips were layered on 13% (w/v) acrylamide gels and sealed with agarose [0.65 % (w/v) in 1x electrode buffer] for separation in second dimension using Ruby SE600 electrophoresis set up (GE healthcare). SDS-PAGE was carried out at 20 mA for initial 1 h and 30 mA for next 3.5 h or till bromophenol blue dye reached the end of the gel. Following SDS-PAGE, the gels were rinsed with MilliQ water 2-3 times, fixed for 1 h in methanol/water/acetic acid (5:4:1, v/v/v) and stained for 16-18 h in colloidal Coomassie stain [0.08% (w/v) Coomassie Brilliant Blue G-250, 20% (v/v) ethanol, 8% (w/v) ammonium sulphate and 0.35 M phosphoric acid]. Gels were destained for 24 h in deionised water with several changes on a shaker and after completion of destaining, gels were stored in 20% (v/v) methanol.

Image visualization and data analysis
A total of eighteen gels (2 genotypes, three ripening stages and three biological replicates at every ripening stage) were scanned using Image Scanner (GE Healthcare) and images were analyzed with Image Master 2D Platinum Software 6.0 (GE healthcare) as per manufacturer’s instructions. About 800 protein spots were detected and matched. Spots which matched automatically in all the gels in both wild type and hp1 were also manually verified. The percent spot volumes were statistically analyzed using one-way ANOVA (Sigmaplot version 11.0) and the spots showing 2-fold differences (up- and down-regulation in at least one of the stages during ripening) in all three biological replicates with a p-value of ≤0.05 were selected for identification by mass spectrometry. The power of test was determined as 0.9-0.99 using one-way ANOVA, from the percent spot volumes.

**Trypsin digestion**

The spots selected for further identification by MALDI-TOF/TOF analysis were manually excised and digested with trypsin (sequencing grade, Promega, Wisconsin, USA) as previously described (Shevchenko et al., 2006). Eluted peptide fragments were concentrated in a speed vacuum concentrator (Thermo Fisher Scientific, MA, USA) and were reconstituted in 5 μL of 1:1 (v/v) acetonitrile (ACN) and 1% (v/v) trifluoroacetic acid (TFA).

**Mass Spectrometry**

2 μL of peptide fragments obtained after trypsin digestion was mixed with 2 μL of α-cyano-4-hydroxycinnamic acid matrix [freshly made in 50% (v/v) ACN and 1% (v/v) TFA (1:1 (v/v))] and spotted onto MALDI target plate and dried. MS/MS analyses were carried out using AutoFlex III smartbeam MALDI TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). TOF spectra within the range of 500 to 4000 m/z were obtained using Flex Control software 3.0 (Bruker Daltonics) in the reflector mode with a laser intensity of 6000 V and an average of 2000 shots per spectrum. The mass spectra were calibrated with external standards with masses ranging from 757.399 m/z to 3147.4710 m/z. Trypsin autolysis peaks were used for internal calibration and they are as follows: trypsin (108-115; m/z-842.509400), trypsin (98-107; m/z-1045.563700), trypsin (58-77; m/z-2211.104000), trypsin (78-97; m/z-2283.180200), trypsin (58-72; 1713.808400), trypsin (62-77; 1774.897500), and trypsin (58-76; m/z-2083.009600). A range of 2-10 precursor ions were selected for MS/MS analysis. Mono-isotopic mass was attributed using Bruker’s SNAP procedure by collecting data from an average of 5000 laser shots at a threshold intensity of 2,500 over the mass range. Peak lists from MS and MS/MS analyses were generated using Flex Analysis software 3.0 (Bruker Daltonics). Proteins were identified using MASCOT version 2.4.01
Matrix Science, Boston, MA) with the help of Biotools software (Bruker Daltonics). The following parameters were set for searches against target databases such as NCBInr (downloaded on 1st Oct, 2012; 20543454 sequences, 7050788919 residues), SwissProt (downloaded on 1st Oct, 2012, 537505 sequences, 190795142 residues) and Plant EST (downloaded on 1st Oct, 2012, 157998786 sequences, 27903646510 residues):

1. tryptic cleavage with a maximum of 2 missed cleavages, 
2. mass tolerance of 30-100 ppm, 
3. Peptide mass tolerance of 0.1-1.0 Da 
4. a minimum of two peptides matching to the protein, 
5. fixed and variable modifications like carbamidomethylation of cysteine and methionine oxidation respectively. In addition, we also searched in SGN-solanaceae genome network (offline from Mascot) at the link ftp://ftp.solgenomics.net/genomes/Solanum_lycopersicum/annotation/ITAG2.3_release/ITA G2.3_proteins.fasta. The false discovery rate (FDR) for the statistically significant proteins was found to be 5% using BH method (Benjamini and Hochberg, 1995; Eravci et al., 2009).

Hierarchical clustering

PermutMatrix software version 1.9.3 was used to generate hierarchical clustering (Pearson’s algorithm) (Caraux and Pinloche 2005). The data file with all identified proteins together with their expression profiles were given as input, and a cluster map along with protein expression profile was generated.

RNA isolation and quantitative Real Time PCR

RNA was isolated from outer pericarp of wild type and hp1 fruits at three ripening stages viz. MG, BR and RR (each with 3 biological replicates) using TRI reagent (Sigma-Aldrich, T9424) as per manufacturer’s protocol. The isolated RNA was treated with RQ1 RNase Free DNase (Promega) as per manufacturer’s recommendations to remove DNA. Reverse transcription was performed with 2 µg of RNA using SuperScript® III RT First Strand Synthesis Kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer’s protocol. qRT-PCR was performed with about 4 ng of total RNA in 10 µl of reaction volume using SYBR Green PCR Master Mix (Takara, Japan) on a 7300 Fast Real Time PCR System (Applied Biosystems, http://www.appliedbiosystems.com/). Gene specific primers were designed from the sequences obtained from SGN database using Primer 3 software and represented in Table S3. The relative fold differences for each sample were determined by normalizing Ct values of each gene to the mean expression of both β-Actin and Ubiquitin genes and were calibrated using the formula $2^{-\Delta Ct}$ which gives the relative expression of each gene. Mean±SE
(Sigmaplot version 11.0) of Ct values obtained from three biological replicates were used for calculation of expression levels.

**Western Blotting**

The outer pericarp of tomato fruit (from each genotype, three biological replicates were used) was homogenized in extraction buffer [200 mM Tris pH 8.0, 150 mM NaCl, 2.5 μL protease inhibitor cocktail per mL extraction buffer (Sigma, MO, USA), 1 mM PMSF and 0.125% (v/v) Triton X-100] and centrifuged at 13,000g at 4°C for 15 min and the supernatant was collected. The protein content was quantified by Bradford’s method (Bradford 1976). After electrophoresis on 12% SDS-PAGE (Laemmli 1970), proteins were electroblotted onto PVDF membrane (Millipore) (Towbin et al., 1979). The blots were incubated with 1:2500 dilution of antibodies raised against bell pepper PAP protein followed by anti-rabbit IgG alkaline phosphatase conjugate (1:80,000 dilution, Sigma-Aldrich). The blots were finally developed using chromogenic substrates for alkaline phosphatase- 66 μL of NBT (nitroblue tetrazolium, 50 mg/mL) and 33 μL of BCIP (5-bromo-4-chloro-3-indolyl phosphate, 50 mg/mL) in 10 mL of buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl₂, pH 9.5) (Sreelakshmi 2000).

**Determination of Ethylene Evolution**

To measure ethylene evolution after harvesting, fruits were enclosed in air tight boxes (250 mL). After 4 hours, 1 mL of head space gas was removed from the boxes and injected into a Porapak T column connected to a gas chromatograph (GC-17A, Shimadzu) as previously described (Santisree et al., 2011). For every sample, ethylene in the headspace gas was measured at least three times using a minimum of three biological replicates at each ripening stage.

**Estimation of Carotenoids**

Carotenoids were extracted from fruits and estimated using a previously described procedure (Barba et al., 2006). Carotenoids were separated and analysed by HPLC (Shimadzu, Japan) on a C-18 column (250 X 4.6 mm; 5 micron, Phenomenex, Torrance, CA) using a 60 min isocratic gradient of methanol:acetonitrile (90:10, v/v) + triethylamine (TEA, 9 μM) as mobile phase. Carotenoids were identified and quantified based on the retention times and peak areas of the standards (lycopene, L-9879 from tomato, β-carotene, C-4582, lutein, X-6250, Sigma-Aldrich) and β-carotene, lutein and lycopene contents were determined by monitoring absorbance at 475 nm (Barba et al., 2006) using a UV-VIS detector (SPD 20 A, Shimadzu).
Isolation of plastoglobules and Western blotting

Plastoglobules were isolated from tomato fruits at RR stage using a method adapted from two protocols; the procedure of Besagni et al., 2011 and that of Lundquist et al., 2012. About 400 g of fruit tissue was ground in 400 mL of cold homogenisation buffer [450 mM sorbitol, 20 mM Tricine–KOH, pH 8.4, 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaHCO₃, 1 mM MnCl₂, 5 mM Na-ascorbate, 0.05% (w/v) bovine serum albumin (BSA) fraction V, and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The homogenate was filtered immediately through 4 layers of 20 μm miracloth and the filtrate was distributed equally into eight 50 ml tubes and then centrifuged for 10 min at 1,200 g. Each chromoplast membrane pellet was gently resuspended in 4-5 mL of 0.6 M sucrose/1x TrE [10× stock solution has 50 mM Tricine–KOH, pH 7.5, 2 mM EDTA, and 2 mM dithiothreitol (DTT)] and these were pooled into a 50-mL Falcon tube. The membrane fraction was then sonicated 5x 40s on ice at an amplitude of 23% with a 3 min interval in between each sonication (Sonics and Materials, Inc. Vibracell VCX 130PB). The samples were disbursed in 5-6 mL aliquots into the required number of UltraClear SW28 tubes, and were carefully overlaid with the sucrose/1x TrE solutions in the following order: 6 mL of 38% (w/v) sucrose, 6 mL of 20% (w/v) sucrose, 4 mL of 15% (w/v) sucrose, and finally 8 mL of 5% (w/v) sucrose to the top of the tube. These gradients were centrifuged for 16 h in a Beckman Coulter Optima L-90K centrifuge at 100,000 g. The plastoglobule rich fraction was then collected from the top layer.

The plastoglobule fraction thus obtained was dialysed against 1x TrE buffer using a 3500 MWCO membrane (Thermo Scientific, Rockford, USA) and the protein was precipitated from the plastoglobule fraction using methanol-chloroform as essentially described in Besagni et al., 2011. After precipitation, the protein was resuspended in sample buffer, boiled and then separated on a 12% SDS-PAGE followed by Western blotting with CHRC antibody as described above.

Confocal microscopy of isolated plastoglobules

Confocal images of purified plastoglobules were obtained with a laser scanning confocal microscope (Carl Zeiss LSM 710 NLO, Jena, Germany) coupled to an inverted microscope (with NDD multiphoton laser). Freshly isolated plastoglobules were placed on a glass slide and covered with a coverslip. The carotenoid autofluorescence from the isolated plastoglobules was examined by measuring the emission between 500-510 nm (green) and
chlorophyll emission was measured between 740-750 nm (red) after excitation with a laser at 488 nm.

In addition, the absorption spectrum for carotenoids from the plastoglobule fraction was obtained as described below. To about 100 μl of freshly isolated plastoglobule fraction, an equal volume of n-hexane was added and mixed thoroughly. The mixture was centrifuged at 12000g for 5 min and the supernatant was dried in a speed vac. After drying, the sample was dissolved in 6 μl of n-hexane and the absorption spectra for 1.5 μl x3 were recorded in a Nanodrop spectrophotometer (ND1000, Thermo Scientific) from 350-700 nm.

**Supplemental data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Randomized block design describing growth of plant material, collection of fruit tissue at different stages of ripening in three biological replicates for 2D electrophoresis and protein identification.

**Supplemental Figure S2.** Visualization of outer pericarp in green and red fruits of tomato. Outer pericarp thickness was measured using Vernier Caliper.

**Supplemental Figure S3.** Functional classification of differentially expressed proteins isolated from outer pericarp of tomato fruits.

**Supplemental Figure S4.** Schematic representation of metabolic pathways depicting the exchange of metabolites between different subcellular compartments and organelles in tomato fruits. The differentially expressed proteins in WT (green) and hp1 (red) are shown at relevant places in the pathway. The relative abundance of proteins is depicted in bar diagram (with height of bar denoting extent of expression) sequentially at MG, BR and RR stages of ripening in both WT (green) and hp1 (red) fruits.

**Supplemental Figure S5.** Schematic representation of electron transport chain operating in the thylakoid membranes of chloroplasts. The electrons produced by splitting of water molecule in the lumen are transported through PSII to PSI to generate proton motive force necessary for production of ATP and NADPH. Chromoplast specific carotenoid associated
protein is also present in the membrane and binds to carotenoids. Fe-SOD participates in detoxification of superoxide free radicals generated from light reactions. The abundance of proteins at different stages (Fig. 4) is depicted for WT (green) and \textit{hp1} (red).

**Supplemental Figure S6.** Hypothetical model representing the induction of Hsps during fruit ripening and their probable role in tomato fruits.

The pool of Reactive Oxygen Species (ROS) produced from various metabolic processes is detoxified by ascorbate peroxidase and superoxide dismutase. ROS also induces the production of both large and small heat shock proteins which together with the help of 14-3-3 proteins and peptide methionine sulfoxide reductase (PMSR) may participate in preventing the denaturation of proteins and the formation of insoluble protein aggregates. Heat shock proteins may also interact with ankyrin 2A and 2B proteins and may aid in targeting of chloroplast proteins to outer membranes (Kim et al., 2011). The abundance of proteins at different stages (Fig. 4) is depicted for WT (green) and \textit{hp1} (red).

**Supplemental Figure S7.** Comparison of protein, transcript and metabolite levels in WT and \textit{hp1} fruits during ripening.

**Supplemental Figure S8.** Homology between sequences of different chromoplast associated proteins.

**Supplemental Table S1.** Identification of differentially expressed proteins in both WT and \textit{hp1} fruits by MALDI-TOF-TOF analysis.

**Supplemental Table S2.** The differentially expressed proteins (Table S1) identified in this study were compared with other studies (Rocco et al., 2006; Faurobert et al., 2007; Qin et al., 2012) to identify similarities and differences in the proteome complement. The table lists the proteins that are specifically identified in the current study.

**Supplemental Table S3.** Primers used in the current study for quantitative real time PCR analysis.

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**Figure legends**

**Figure 1.** Characterization of the phenotypic and metabolic differences between WT and hp1 fruits. A, Phenotypes of WT and hp1 fruits at mature green (MG), breaker (BR) and red ripe (RR) stages during ripening. B, Levels of lycopene (left) and β-carotene (right) in red ripe fruits of WT and hp1 (n>3±SE). C, Lutein levels in RR fruits of hp1 and WT (n>3±SE). D, Ethylene emission from WT and hp1 fruits at different stages of ripening (n>3±SE). E, Comparison of pericarp proteome profiles isolated from WT and hp1 fruits at MG, BR and RR stages. The representative pictures of 2-DE gels are shown.

**Figure 2.** A representative 2-DE colloidal coomassie blue stained gel showing profile of proteins extracted from hp1 fruits at BR stage. The numbers indicate the differentially expressed proteins that were later identified by mass spectrometry and the arrows indicate their relative positions in the gel.

**Figure 3.** Hierarchical clustering analysis of differentially expressed proteins in WT and hp1 fruits during ripening. A, Using Permut Matrix 1.9.3, proteins were clustered into three clusters based on their percent spot volumes. Color corresponding with relative level of expression is given on top of heat map. The identity of protein (spot no.) is indicated on the right side of the rows. The columns represent levels of proteins at MG, BR and RR stages of WT and hp1 fruits respectively. B, Pie diagram showing relative distribution of different functional classes of proteins in each cluster.

**Figure 4.** The biosynthetic pathway of carotenoids depicting the expression profiles of the enzymes catalyzing different steps beginning from the precursors. The dotted arrows indicate multiple steps in the pathway. The transcripts were quantified in WT and hp1 at MG, BR and RR stages of fruit development using real time PCR. The levels of transcripts were expressed after normalization with two internal controls, β-actin and ubiquitin.

Abbreviations: GA3-P- Glyceraldehyde-3-phosphate, DXP- Deoxy-xylulose 5-phosphate, IPP- Isopentenyl pyrophosphate, DMAPP- Dimethylallyl pyrophosphate, GGDP- Geranyl geranyl diphosphate, DXS- Deoxy-xylulose 5-phosphate synthase, IDI- Inositol diphosphate synthase, GGDS-2- Geranyl geranyl diphosphate synthase2, PSY1- Phytoene synthase1, PSY2- Phytoene synthase 2, PDS- Phytoene desaturase, ZIS- ζ-carotene isomerase, CRTISO- Carotenoid isomerase, ZDS- ζ-carotene desaturase, LCYB1- Lycopene β-cyclase 1, LCYB2-
Lycopene β-cyclase 2, CYCB- Chromoplast specific Lycopene β-cyclase, LCYE- Lycopene ε-cyclase, CRTRB2- Beta carotene hydroxylase 2, ZEP- Zeaxanthin epoxidase, VDE- Violoxanthin deepoxidase, CRTRB1- Beta carotene hydroxylase 1, CYP97A29- Cytochrome P450 carotenoid β-hydroxylase A29, CYP97C11- Cytochrome P450 carotenoid ε-hydroxylase C11.

**Figure 5.** Comparison of chromoplast-associated carotenoid binding protein levels in WT and *hp1* fruits. Unless specifically indicated, equal amount of protein was loaded in all the immunoblots. A, Relative spot volume of CHRC protein in WT and *hp1* fruits at different stages of ripening. B, Real time PCR quantification of CHRC transcript in WT and *hp1* fruits at different stages of ripening. C, Immunoblotting of proteins from bell pepper and tomato fruits. The polyclonal antibody raised against bell pepper PAP recognized a 35±2 kD protein in tomato. Since PAP levels were very high in bell pepper, a reduced amount of protein (0.3 μg) was loaded compared to WT tomato fruits (10 μg). D, Comparison of CHRC levels in WT and *hp1* fruits at RR stage. Progressively increasing concentration of proteins was loaded on the gel. The equivalence in the band intensity of *hp1* (2.5 μg) with that of WT (5.0 μg), indicates that CHRC level in *hp1* is ca. 2-fold higher than WT. E, Comparison of CHRC levels in WT and *hp1* fruits at MG and RR stages. Ripening stimulated an increase in the level of CHRC protein from MG to RR stage, with higher CHRC levels in *hp1* fruits. F, Comparison of CHRC levels in RR fruits of AC, AV and *nor*. Note that *nor* mutant though accumulates very little lycopene, exhibits normal level of CHRC equal to other two cultivars. G, Relative abundance of CHRC protein in *hp1* and *hp2* fruits at MG stage. H, Relative levels of CHRC in *hp1* and *hp2* and *Ip* fruits at RR stage. Note that *hp1* and *hp2* exhibit similar level of CHRC, while *Ip* showed intermediate level of CHRC between WT and *hp1*, *hp2* fruits. I, Analysis of CHRC levels in RR fruits of WT, *hp3* and *hp1*. The order of abundance of CHRC is *hp1>*hp3>*WT. J, Comparison of CHRC transcript levels in the RR fruits of *hp1*, *hp2*, *hp3* and *Ip* in relation to WT. Note that all the mutants exhibit high CHRC transcript level compared to WT.

**Figure 6.** Confocal microscope imaging and Western blotting of isolated plastoglobules. A, Sucrose density gradient purification of plastoglobules. Note the distinct upper layer of purified plastoglobules is well separated from the underlying chromoplast membranous fractions. B-D, Purified plastoglobules were visualized using a confocal microscope. On
excitation with a 488 nm laser the plastoglobules exhibit strong green fluorescence (B) (emission in between 500-510 nm) but show negligible red fluorescence (emission in between 740-750 nm) D, Bright field image of plastoglobules. E, Enlarged view of a single plastoglobule. Note the green fluorescence emitted from plastoglobules is uniformly distributed. F, Absorption spectrum of the extract obtained from purified plastoglobules showing characteristic carotenoids peak at 440-475 nm. G, Western analysis of CHRC levels in the purified plastoglobule fraction (PG-plastoglobule, 0.33 μg) in comparison to chromoplast membrane fraction (CF-chromoplast fraction prior to sonication, 2.5 μg) and total protein from the outer pericarp of RR fruits of hpI (FE-fruit extract, 2.5 μg). Note that the plastoglobule fraction shows enrichment of CHRC protein.
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Figure 4. The biosynthetic pathway of carotenoids depicting the expression profiles of the enzymes catalyzing different steps beginning from the precursors. The dotted arrows indicate multiple steps in the pathway. The transcripts were quantified in WT and hp1 at MG, BR and RR stages of fruit development using real time PCR. The levels of transcripts were expressed after normalization with two internal controls, β-actin and ubiquitin.

Abbreviations: GA3-P- Glyceraldehyde-3-phosphate, DXP- Deoxy-xylulose 5-phosphate, IPP-Isopentenyl pyrophosphate, DMAPP- Dimethylallyl pyrophosphate, GGDP- Geranyl geranyl diphosphate, DXS- Deoxy-xylulose 5-phosphate synthase, IDI- Inositol diphosphate synthase, GGDS2-Geranyl geranyl diphosphate synthase2, PSY1- Phytoene synthase1, PSY2- Phytoene synthase 2, PDS-Phytoene desaturase, ZIS- ζ-carotene isomerase, CRTISO- Carotenoid isomerase, ZDS- ζ-carotene desaturase, LCYB1- Lycopene β-cyclase 1, LCYB2- Lycopene β-cyclase 2, CYCB- Chromoplast specific Lycopene β-cyclase, LCYE- Lycopene ε-cyclase, CRTRB2- β-carotene hydroxylase 2, ZEP-Zeaxanthin epoxidase, VDE- Violoxanthin deepoxidase, CRTRB1- β-carotene hydroxylase 1, CYP97A29- Cytochrome P450 carotenoid β-hydroxylase A29, CYP97C11- Cytochrome P450 carotenoid ε-hydroxylase C11.
**Figure 5.** Comparison of chromoplast-associated carotenoid binding protein levels in WT and *hp1* fruits. Unless specifically indicated, equal amount of protein was loaded in all the immunoblots. A, Relative spot volume of CHRC protein in WT and *hp1* fruits at different stages of ripening. B, Real time PCR quantification of CHRC transcript in WT and *hp1* fruits at different stages of ripening. C, Immunoblotting of proteins from bell pepper and tomato fruits. The polyclonal antibody raised against bell pepper PAP recognized a 35±2 kD protein in tomato. Since PAP levels were very high in bell pepper, a reduced amount of protein (0.3 μg) was loaded compared to WT tomato fruits (10 μg). D, Comparison of CHRC levels in WT and *hp1* fruits at RR stage. Progressively increasing concentration of proteins was loaded on the gel. The equivalence in the band intensity of *hp1* (2.5 μg) with that of WT (5.0 μg), indicates that CHRC level in *hp1* is 2-fold higher than WT. E, Comparison of CHRC levels in WT and *hp1* fruits at MG and RR stages. Ripening stimulated an increase in the level of CHRC protein from MG to RR stage, with higher CHRC levels in *hp1* fruits. F, Comparison of CHRC levels in RR fruits of AC, AV and *nor*. Note that *nor* mutant though accumulates very little lycopene, exhibits normal level of CHRC equal to other two cultivars. G, Relative abundance of CHRC protein in *hp1* and *hp2* fruits at MG stage. H, Relative levels of CHRC in *hp1* and *hp2* and *Ip* fruits at RR stage. Note that *hp1* and *hp2* exhibit similar level of CHRC, while *Ip* showed intermediate level of CHRC between WT and *hp1*, *hp2* fruits. I, Analysis of CHRC levels in RR fruits of WT, *hp3* and *hp1*. The order of abundance of CHRC is *hp1>*hp3>*WT. J, Comparison of CHRC transcript levels in the RR fruits of *hp1*, *hp2*, *hp3* and *Ip* in relation to WT. Note that all the mutants exhibit high CHRC levels compared to WT.
Figure 6. Confocal microscope imaging and Western blotting of isolated plastoglobules. A, Sucrose density gradient purification of plastoglobules. Note the distinct upper layer of purified plastoglobules is well separated from the underlying chromoplast membranous fractions. B-D, Purified plastoglobules were visualized using a confocal microscope. On excitation with a 488 nm laser the plastoglobules exhibit strong green fluorescence (B) (emission in between 500-510 nm) but show negligible red fluorescence (emission in between 740-750 nm) D, Bright field image of plastoglobules. E, Enlarged view of a single plastoglobule. Note the green fluorescence emitted from plastoglobules is uniformly distributed. F, Absorption spectrum of the extract obtained from purified plastoglobules showing characteristic carotenoids peak at 440-475 nm. G, Western analysis of CHRC levels in the purified plastoglobule fraction (PG-plastoglobule, 0.33 μg) in comparison to chromoplast membrane fraction (CF-chromoplast fraction prior to sonication, 2.5 μg) and total protein from the outer pericarp of RR fruits of hp1 (FE-fruit extract, 2.5 μg). Note that the plastoglobule fraction shows enrichment of CHRC protein.