Chromoplast-Specific Carotenoid-Associated Protein Appears to Be Important for Enhanced Accumulation of Carotenoids in hp1 Tomato Fruits\textsuperscript{1[C][W][OA]}

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Tomato (\textit{Solanum lycopersicum}) high-pigment mutants with lesions in diverse loci such as DNA Damage-Binding Protein1 (\textit{hp1}), Deetiolated1 (\textit{hp2}), Zeaxanthin Epoxidase (\textit{hp3}), and Intense pigment (\textit{lp}; 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 \textit{hp1} mutant and wild-type (cv Ailsa Craig) 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 \textit{hp1} fruits at red-ripe stage that is also reflected in its transcript level. Western blotting using CHRC polyclonal antibody from bell pepper (\textit{Capsicum annuum}) revealed a 2-fold increase in the abundance of CHRC protein in the red-ripe stage of \textit{hp1} fruits compared with the wild type. CHRC levels in \textit{hp2} were found to be similar to that of \textit{hp1}, whereas \textit{hp3} and \textit{lp} showed intermediate levels to those in \textit{hp1}, \textit{hp2}, and wild-type fruits. Both CHRC and carotenoids were present in the isolated plastoglobules. Overall, our results suggest that loss of function of \textit{DDB1}, \textit{DET1}, \textit{Zeaxanthin Epoxidase}, and \textit{lp} up-regulates CHRC levels. 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.

\textsuperscript{1} This work was supported by the Department of Biotechnology, Government of India (grant no. BT/PB1671/PBD/16/828/2008 to Y.S. and R.S.), the University Grants Commission, New Delhi (junior research fellowship to H.V.K.), and the Council of Scientific and Industrial Research, New Delhi (senior research fellowship to R.K.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.112.212191
capacity for the carotenoids and/or pigments (Liu et al., 2004; Kolotilin et al., 2007). Likewise, the deficiency of abscisic acid (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 during the conversion of chloroplasts to chromoplasts. The first is the disintegration of thylakoid membranes, followed by loss of chlorophyll, increase in the number of plastoglobules, accumulation of lycopene, and 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 the fibrillar form, as observed in bell pepper (Capsicum annuum; Pozueta-Romero et al., 1997). In addition, these plastoglobules also appear to be involved in regulating carotenoid metabolism, as enzymes such as \( \zeta \)-carotene desaturase, lycopene \( \beta \)-cyclase, and \( \beta \)-carotene hydroxylases are present in them (Ytterberg et al., 2006). Furthermore, proteins called plastoglobulins are associated with plastoglobules and help in the accumulation of carotenoids. These include fibrillin, also known as CHRB or plastid lipid-associated protein (PAP) from pepper chromoplast fibrils, and chromoplast-associated carotenoid-binding protein (CHRC) and CHRD from petals of cucumber (Cucumis sativus; 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 (Giuliano et al., 1993; Vishnevetsky et al., 1999).

All the plastoglobulins share a hydrophobic domain of 17 to 19 amino acids, and this region seems to be important for carotenoid-protein interactions (Vishnevetsky et al., 1999). The significance of the above-mentioned plastoglobulins in the sequestration and storage of carotenoids was elegantly exemplified in two studies (Leitner-Dagan et al., 2006; Simkin et al., 2007). Overexpression of a pepper fibrillin gene in tomato resulted in a 2-fold increase in carotenoid levels as well as carotenoid-derived volatiles (Simkin et al., 2007), whereas 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-overexpressing tomato (Simkin et al., 2007). In addition, the Or gene of cauliflower (Brassica oleracea), which encodes a DnaJ Cys-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 alternative strategy to increase carotenoid levels in food crops.

Currently, using systems biology approaches, efforts are being 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 \( \textit{rin}, \textit{nor}, \) and \( \textit{Nr} \) mutants led to a comprehensive understanding of ethylene-regulated processes during ripening and also reinforced the need for the integration of transcript, proteome, and metabolite analyses (Osorio et al., 2011). In the \( \textit{hp1} \) mutant, transcription factor profiling was combined with microarray and metabolite analyses, and that study revealed that the secondary metabolism is controlled at the transcriptional level (Rohrmann et al., 2011). Using fruit-specifically down-regulated \( \textit{DETI} \) 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) as well as other fruits like strawberry (\( \textit{Fragaria} \) spp.; Bianco et al., 2009), grape (\( \textit{Vitis} \) \( \textit{vinifera} \); Zhang et al., 2008), and \( \textit{Citrus} \) spp. (Zeng et al., 2011) and also in isolated chromoplasts (Siddique et al., 2006; Barsan et al., 2010). This led 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 given the importance of the \( \textit{hp1} \) mutant for improving the phytonutrient quality, we undertook a proteomic approach to decipher the role of DDB1 in tomato fruit ripening, as information on the proteome complement of \( \textit{hp1} \) is lacking. We compared the proteome profiles of outer pericarp in both wild-type and \( \textit{hp1} \) fruits at different stages during ripening. Although we did not find significant differences between the wild type and the mutant, we found a development-specific fine-tuning in the abundance of several proteins controlling multiple metabolic pathways in both wild-type and \( \textit{hp1} \) fruits during ripening. We found higher levels of CHRC in \( \textit{hp1} \). Our results suggest a likely relationship between increased lycopene content and CHRC levels during fruit ripening.

RESULTS AND DISCUSSION

Influence of DDB1 on the Tomato Proteome

\textit{High pigments} fruits are characteristically different from wild-type fruits in being more green in color at the mature green (MG) stage (Fig. 1A) and accumulating more lycopene at the red-ripe (RR) stage (Fig. 1, A and B). In view of this, we characterized the proteome profiles at both these stages. We also included the breaker (BR) stage, where the fruit makes transition, marking the onset of the ripening process. Consistent with earlier reports, loss of function of \( \textit{DDB1} \) led to an 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). In contrast, ethylene, an important regulator of fruit ripening, although elevated in hp1 at the 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 three biological replicates was independently carried out according to the scheme shown in Supplemental Figure S1. For proteome profiling, we analyzed the proteins extracted from the outer pericarp of wild-type and hp1 fruits (Supplemental Fig. S2) at MG, BR, and RR stages. Representative two-dimensional gel electrophoresis images comparing the above three stages in wild-type and hp1 fruits are shown in Figure 1E. Image analysis of the gels (n = 3) with the Image Master Platinum software revealed about 800 proteins on each gel. Seventy-two proteins were found to be differentially expressed by at least 2-fold (overexpression or underexpression; statistically significant expression based on ANOVA with P ≤ 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 hp1 fruits, we did not find any proteins present specifically in the wild type or the mutant alone. This indicates that the subtle changes in proteome profile may be responsible for the specific phenotype of hp1 fruits. To ascertain this, we identified the molecular nature of differentially expressed proteins.

Figure 1. Characterization of the phenotypic and metabolic differences between wild-type and hp1 fruits. A, Phenotypes of wild-type (WT) and hp1 fruits at MG, BR, and RR stages during ripening. B, Levels of lycopene (left) and β-carotene (right) in RR fruits of the wild type (WT) and hp1 (n > 3). C, Lutein levels in RR fruits of hp1 and the wild type (WT; n > 3). D, Ethylene emission from wild-type (WT) and hp1 fruits at different stages of ripening (n > 3). FW, Fresh weight. Error bars indicate SE. E, Comparison of pericarp proteome profiles isolated from wild-type and hp1 fruits at MG, BR, and RR stages. Representative images from two-dimensional gel electrophoresis are shown. [See online article for color version of this figure.]
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 (Caraux and Pinloche, 2005) using the differences in protein spot intensity at different ripening stages of both wild-type and hp1 fruits. The above analysis distinguished three main clusters of proteins, hereafter named clusters I, II, and III (Fig. 3A). As expected, in all 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 fruit development. This subcluster included 14 spots, of which the identity of six (3, 10, 37, 47, 48, and 70) was not known. The remaining eight spots (2, 8, 11, 12, 13, 42, 43, and 52) belonged to proteins involved in carbohydrate metabolism. The second subcluster consisted of proteins whose level was similar at the MG and RR stages but varied at the BR stage. This cluster consisted of five proteins, of which two were unidentified proteins (34 and 44) and the remaining three were involved in carbohydrate metabolism (5, 9, and 17). The third subcluster comprised three proteins that declined during fruit development (1, 6, and 7). While the majority of these proteins belonged to housekeeping functions, 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 the BR stage in the wild type (but with almost similar expression levels at MG and RR) but were up-regulated in hp1 and were specifically involved in carbohydrate metabolism (21, 23, 25, 29, 30, and 45). The proteins that showed higher expression in the BR stage of hp1, with reduced but similar expression levels at MG and RR, were classified in subcluster 2 (15, 19, 22, 28, 36, 38, 50, and 51). While most of these proteins were part of the photosynthesis machinery, this also included a small heat shock protein17.7 (50). Subcluster 3 consisted of proteins that showed almost constant expression levels in both the wild type and hp1 (16, 18, 20, 24, 32, 33, and 63) during ripening. These proteins belonged to carbon metabolism (24 and 32), amino acid/hormone metabolism (18 and 33), cell cycle regulation (16), secondary metabolism (20), transport (40), and detoxification (63; Fig. 3B).

Cluster III (36.12% of proteins) comprised proteins with diverse biological functions showing similar expression patterns in either the wild type or hp1.
**Figure 3.** Hierarchical clustering analysis of differentially expressed proteins in wild-type (WT) and *hp1* fruits during ripening. 

A, Using PermutMatrix 1.9.3, proteins were clustered into three clusters based on their percentage spot volumes. A color bar
across different developmental stages during ripening. In wild-type fruits, this cluster included proteins modulating stress (60, 62, 65, and 68), detoxification (49), hormone metabolism (41), secondary metabolism (58), and unknown functions (54, 57, 71, and 72; Fig. 3B). The spots that were specifically modulated in hp1 fruit consisted of proteins involved in protein fate modification/degradation (46 and 56), detoxification (49), stress response (61), and a few of unknown function (39, 57, and 71; Fig. 3B). One of the spots (66) showed a diametrically different expression pattern in the wild type and hp1. It had higher expression in the wild type at the 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 fruit ripening revealed three main pathways. The first pathway comprised proteins that are involved in the Calvin-Benson cycle, glycolysis, the tricarboxylic acid cycle, and the interconnecting pathways (Supplemental Fig. S4). 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 an Accumulation of Proteins Related to Carotenoid Sequestration and Metabolism**

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

One protein that showed an interesting expression pattern is CHRC. This protein showed a gradual decline in abundance during ripening in the wild type. 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 chromoplast proteome studies of tomato, bell pepper, and *Citrus* spp. (Siddique et al., 2006; Barsan et al., 2010; 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**

Although it is logically expected that high carotenoid levels in *hp1* fruits should be reflected 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 the wild type 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 the *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 involved in carotenoid synthesis using quantitative real-time PCR analysis, including genes contributing to the formation of the first precursor phytoene. Despite the accumulation of higher levels of lycopene in *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 the BR and RR stages when compared with the wild type. The absolute levels of different transcripts varied distinctly and could be classified into two groups. In wild-type fruit at the MG stage, *LCYB1, LCYB2, ZEP,* and *NCED1* were the most abundant transcripts (greater than 1.0–8.0), whereas...
other genes were expressed at a lower level (0.0–1.0). A comparison of wild-type gene expression with hp1 fruit at the MG stage indicated that the expression of most genes was significantly down-regulated in the mutant, except CYP97A29, CYP97C11, CRTRB1, and CRTRB2, which were up-regulated.

In the wild type, GGDS2 and LCYB2 were the most abundant transcripts (greater than 1.0–13.0) at the BR stage, whereas other genes were expressed at a lower level. While the expression levels of DXS, IDI, GGDS2, PSY1, ZDS, and VDE were nearly similar, other transcripts, such as PSY2, ZIS, CRTISO, LCYB2, CYCB,
that may regulate down-regulated tomato fruits. One of the enzymes and changes in the carotenoid levels in between transcript pro biosynthesis pathway, as they did not the posttranscriptional regulation of the carotenoid Similar to this study, Enhanced carotenoid formation in the hp1 shows up-regulation at the RR stage, indicates that the barring a few such as HP1, higher lutein levels found in RR fruits of hp1, the transcript levels of most enzymes of the carotenoid biosynthesis pathway were lower than in the wild type. The emergence of “omics” highlights the need for a 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., 2008). The expression of a metabolome phenotype during the development of an organism results from a complex interaction between multiple responses at several levels, from the expression of genes regulating the pathway until the final biosynthesis of metabolites. In order to understand the complexities of the 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 the TOM1 microarray, the transcript profiles obtained at 7 DPA of fruit development were used for normalizing all other data at the different stages (7–57 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.

A Systems Biology Approach Is Required to Uncover the Contribution of hp1 to Fruit Ripening

As an alternative, we compared the transcript profiles for five carotenoid pathway genes obtained in this study with the corresponding metabolite data obtained earlier in the wild type and hp1 at the RR stage (Cookson et al., 2003). These transcript-metabolite pairs include PSY1-phytoene, ZIS-α-carotene, ZDS-lycopene, CRTISO-lycopene, and CYCB-β-carotene. The above pairs were correlated using the Pearson correlation algorithm with XLSTAT 2012 software (Supplemental Fig. S7A). The correlation coefficient values indicated a negative correlation between transcripts and metabolites in the wild type. Despite the high expression of PSY1 and CRTISO in the wild type, low lycopene levels were observed at the 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 increase in phytoene level has a feed-forward effect on carotenoid biosynthesis (Fraser et al., 2007). It was also reported that the activity of the PSY1 enzyme in hp1 was up-regulated by 2-fold, although there was no corresponding increase in the transcripts of PSY1 and PDS (Cookson et al., 2003), suggesting a regulation of PSY1 activity at the posttranscriptional level.

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also observed in the case of DET1 down-regulated tomato lines (Enfissi et al., 2010) and also in PSY1-overexpressing 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 the regulation of metabolic networks in fruit ripening. The negative correlation between carotenoid pathway transcripts and carotenoids indicates the importance of posttranscriptional regulation in elevating carotenoid levels in hp1 fruits. This view is supported by the observation that although there was no corresponding increase in the transcripts of PSY1 and PD5, the activity of the PSY1 enzyme in hp1 was up-regulated by 2-fold (Cookson et al., 2003). A similar lack of correlation was also observed between the transcript profiles of CYP97A29, CYP97C11, CRTRB1, and CRTRB2 and the lutein levels in wild-type and hp1 fruits (Supplemental Fig. S7B). Although the correlation coefficient values obtained were higher in hp1 compared with the wild type, they do not indicate a strong correlation between the transcripts and the lutein levels. The absence of correlation between transcript levels and their respective metabolites (lutein and lycopene) indicate that alternative mechanisms like posttranscriptional regulation might be controlling the carotenoid pathway.

Similarly, we correlated the protein (for five differentially expressed proteins) and the corresponding metabolite data across different developmental stages in both the wild type and hp1 (Rohrmann et al., 2011). The protein-metabolite pairs acid invertase (spot 53)-Glc, UDP-Glc pyrophosphorylase (spot 17)-Suc, SAM synthetase (spot 18)-ethylene, E8 protein (spot 41)-ethylene, and malate dehydrogenase (spot 42)-malate were analyzed by XLSTAT (Supplemental Fig. S7B). Although the correlation coefficient values obtained were higher in hp1 compared with the wild type, they do not indicate a strong correlation between the transcripts and the lutein levels. The absence of correlation between transcript levels and their respective metabolites (lutein and lycopene) indicate that alternative mechanisms like posttranscriptional regulation might be controlling the carotenoid pathway.

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

CHRC was first discovered in cucumber corollas (Smirra et al., 1993) and was found to be a lipid-binding protein. A survey of the literature revealed that this protein was extensively studied in bell pepper (Deruère et al., 1994) and was named 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. S8, A and C). Also, tomato CHRC protein showed 100% sequence similarity to the tomato PAP protein sequence, suggesting that these two proteins are similar (Supplemental Fig. S8A). On the contrary, alignment of tomato CHRC/PAP with tomato fibrillar family members (10 members based on BLAST search in the Solanaceae Genome Network [SGN] database; Supplemental Fig. S8B) revealed a high degree of mismatches except for two sequences (Solyco08g005220.2.1 and Solyco08g076480.2.1), 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 major roles in the storage of carotenoids in chromoplasts (Vainstein et al., 1994; Pozueta-Romero et al., 1997).

Loss of Function of DDB1 Enhances the Abundance of CHRC

A comparison of the protein profiles in wild-type and hp1 fruits revealed an increase in the abundance of CHRC (spot 66) at the RR stage of hp1, whereas its
Therefore, the probability of this protein being recognized by bell pepper antibody is minimal, as an alignment of peptides obtained after MALDI analysis of tryptic peptides from spot 66 showed 100% alignment with CHRC (Supplemental Fig. S8D) but 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 the level of CHRC during ripening is also supported by an increase in its transcript level (Fig. 5, A and B).

A comparison of CHRC protein levels in both wild-type and hp1 fruits at the RR stage revealed at least 2-fold higher CHRC level in hp1 than in the wild type (Fig. 5D). At the MG stage, while the CHRC band was discernible in hp1, in the wild type it was below the detection limit. Immunoblotting indicated that hp1...
fruits show a higher level of CHRC protein, and its level increased during 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 the nor mutant, which accumulates little lycopene, and in cv Arka vikas, an Indian cultivar that has a lycopene level similar to cv Ailsa Craig (AC; the progenitor of the hp1 mutant). Despite the low level of lycopene in nor, it accumulated CHRC protein at a level almost equal to the other two cultivars, Arka vikas 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 chloroplasts purified from mesocarp of a 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 the 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 performed protein profiling from the outer pericarp only.

Since mutation in DET1 (hp2; Mustilli et al., 1999) also leads to a 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 the MG (Fig. 5G) and RR (Fig. 5H) stages revealed an abundance level similar to hp1, suggesting an overlap in the action of hp1 and hp2 loci in regulating CHRC levels. Although DDB1 (hp1) and DET1 (hp2) are diverse loci, the observed overlap may have emanated from their coaction as part of the CUL4-DDB1-DET1 complex (Wang et al., 2008), where loss of function of either of these proteins may contribute to higher CHRC and also pigment levels. Consistent with the enhanced expression of CHRC protein in hp2, Enfissi et al. (2010) showed increases in the number and size of plastoglobules in RR fruits of DET1 down-regulated lines. Similarly, it was reported earlier that the chloroplast-to-chromoplast transition in tomato is accompanied by an increase in the number and size of plastoglobules (Harris and Spurr, 1969). Taken together with 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 the sequestration and stabilization of carotenoids, thus boosting their levels.

The specific role of CHRC in chromoplasts is also supported by the observation that a down-regulation of tomato CHRC using an RNAi strategy led to a 30% reduction of total carotenoids in tomato flowers (Leitner-Dagan et al., 2006). Since RNAi suppression of CHRC reduced the carotenoid level, overexpression should increase its level. Although there are no reports of CHRC overexpression in the literature, overexpression of a related protein, fibrillin from pepper, which shares the PAP domain with CHRC in tomato, resulted in almost a doubling of carotenoid levels and carotenoid-derived aroma compounds in tomato fruits (Simkin et al., 2007). Evidence is emerging for additional roles for plastoglobulin proteins like CHRC, which not only participate in the sequestration of carotenoids but also appear to influence chromoplastogenesis as well as carotenoid metabolism (Brehelin and Kessler, 2008; Li et al., 2012). Taken together, the 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, the Ip mutant exhibits high lycopene content in fruits and also has similar increases 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 a 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 between those in the wild type and hp1 and hp2 fruits (Fig. 5I). Consistent with the intermediate CHRC level, the RR fruits of the Ip mutant also showed intermediate levels of lycopene and β-carotene between those of the wild type and hp1 (Jones, 2000). This suggests that increases in chloroplast number, size, and lycopene level in Ip are likely regulated by a mechanism similar to that in high-pigment mutants and thus shows an intermediate increase in CHRC level.

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 the hp3 mutant revealed a lower abundance of CHRC compared with that in hp1; nonetheless, it was higher than that of the wild type (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. Although hp3 is not a member of the 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 hp1, hp2, hp3, and Ip, it was of interest to examine the transcript levels of CHRC in the RR fruits of these four mutants (Fig. 5J). In comparison with the wild type, hp1 showed the 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 four mutants exhibited high transcript as well as protein levels for CHRC compared with the
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 plastoglobules. The chromoplasts of the hp1 mutant show a 1.3-fold increase in size compared with the wild type (Cookson et al., 2003). Likewise, down-regulation of DET1 leads to an increase in plastoglobe 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 (Austin et al., 2006; Bréhélin and Kessler, 2008). 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 localized in plastoglobules, we examined the presence of carotenoids and CHRC in the isolated plastoglobules. We purified the plastoglobules from RR fruits of tomato by taking advantage of their lower density compared with other membranous fractions on Suc density gradients (Fig. 6A) by adapting the procedures of Besagni et al. (2011) and Lundquist et al. (2012). The purity of the plastoglobule fraction and fluorescence emission from globuli were examined by confocal microscopy (Fig. 6, B–D), which revealed the presence of intact globules (Fig. 6D). Moreover, the purified plastoglobules exhibited a bright green autofluorescence on exposure to a 488-nm argon laser, indicating the enrichment of carotenoids (emission in the range of 500–510 nm; Fig. 6, B and 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 RR fruits, the 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- to 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 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 over the chloroplast membrane fraction or the initial fruit extract. The presence of CHRC in the chloroplast membrane fraction (prior to its sonication to release the plastoglobules) is also in conformity with an earlier transmission electron microscopy observation that the plastoglobules are associated with chloroplast 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 CHRC levels. Several studies have indicated that the CHRC and related proteins, fibrillins, enhance the sequestration of carotenoids into plastoglobules (Bréhélin 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 (Solanum tuberosum) tubers, a massive loss of carotenoids occurs during long-term storage due to enzymatic or nonenzymatic oxidation of carotenoids. However, such loss can be prevented by expression of the 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 the oxidation of carotenoids and ensures the continued formation of carotenoids during the 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 the 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 lp 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.

CONCLUSION

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 the wild type and lp1 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 CHRC by 2-fold by an unidentified mechanism. The lp3 mutant, which is deficient in zeaxanthin epoxidase, also exhibits a higher CHRC level, albeit at a lower level than in lp1 and lp2 mutants. This is consistent with its enlarged plastid compartment size and elevated carotenoid content. The intermediate level of CHRC in lp appears to be related to its intermediate photomorphogenic phenotype and carotenoid levels between the wild type and lp1 and lp2 mutants. Interestingly, transcript profiling of carotenoid pathway genes revealed an overall up-regulation of the pathway in the wild type rather than in lp1, except the desaturation steps catalyzed by PDS and ZDS, suggesting posttranscriptional control of the pathway. Moreover, none of the carotenoid biosynthetic enzymes were detected in our proteomics studies, either because their levels were below the detection limits or they were regulated at the 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 lp1 mutant but not in the wild type, consistent with the elevated lutein levels found in the mutant. Compared with the wild type, transcript levels for ABA biosynthetic genes were down-regulated in lp1, suggesting a relation between low ABA levels and increases in chloroplast number and size, as reported previously in the lp3 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 the enhanced accumulation of carotenoids in high-pigment tomato fruits.

MATERIALS AND METHODS

Plant Material and Experimental Design

Fruits of tomato (Solanum lycopersicum) photomorphogenic mutant lp1 and its isogenic wild type AC (Brinivas et al., 2004) were used for proteomic analysis in this study. The tomato accessions AC (LA2638), lp1 (LA3383), lp2fl (LA2451), Ip (LA1500), hp3 (3-343), and var (LA3770) were obtained from the Tomato Genetics Resource Center (University of California, Davis). Tomato plants were grown under a natural photoperiod in the greenhouse at 28°C ± 1°C during the day and ambient temperature at night. Plants of both the wild type and lp1 were grown in three randomized blocks (each block constitutes one biological replicate), with each block consisting of three rows, with five plants each for the wild type and lp1 per 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 were identified: MG (35 DPA), BR (40 DPA), and RR (49 DPA; Fig. 1A). From each block (biological replicate), 30 to 35 fruits per ripening stage per genotype were harvested, and the outer pericarp was immediately isolated as described below, pooled separately at every ripening stage for both the wild type and lp1 (Supplemental Fig. S1), flash frozen in liquid nitrogen, and stored at −80°C until use.

Plant Material for Protein, Transcript, and Carotenoid Analyses

Fruits of the wild type and lp1 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 the lp1 mutant (Y. Sreelakshmi and R. Sharma, unpublished data), we initiated an independent study on both outer and inner pericarp. The outer pericarp is the outer layer of the fruit including the skin, as indicated in Supplemental Figure S2; its thickness is measured with a Vernier calipers and varies from 0.5 ± 0.1 mm in...
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 Suc, 0.1 M KCl, 0.5 M Tris, pH 7.5, 50 mM EDTA, 50 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 25 μL of protease inhibitor cocktail (Sigma-Aldrich). To this, an equal volume of Tris-saturated phenol was added, and the sample was mixed thoroughly by shaking at 4°C for 30 min. The mixture was centrifuged at 20,000g for 30 min at 4°C. The upper phenolic phase was collected and reextracted two 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 the 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 then once with acetone with 10 mM DTT and finally dissolved in lysis buffer (7 M urea, 2 M thiourea, and 4% [w/v] CHAPS).

The protein was quantified using the Amido Black method (Goldring and Ravaioli, 1996). For each developmental stage, at least three biological replicates (two genotypes, three ripening stages, and three biological replicates at every ripening stage) were scanned using Image Scanner (GE Healthcare). Total numbers of proteins were automatically excised and digested with trypsin (sequencing grade; Promega) as described previously (Shevchenko et al., 2006). Eluted peptide fragments were concentrated in a speed vacuum concentrator (Thermo Scientific) and were reconstituted in 5 μL of 1:1 (v/v) acetonitrile and 1% (v/v) trifluoroacetic acid.

Mass Spectrometry

Peptide fragments (2 μL) obtained after trypsin digestion was mixed with 2 μL of α-cyano-4-hydroxynitrile acid matrix (freshly made in 50% [v/v] acetonitrile and 1% [v/v] trifluoroacetic acid [1:1, v/v]), spotted onto a MALDI target plate, and dried. Tandem mass spectrometry (MS/MS) analyses were carried out using the AutoFlex III smartbeam MALDI-TOF mass spectrometer (Bruker Daltonics). TOF spectra within the range of 500 to 4,000 mass-to-charge ratio (m/z) were obtained using Flex Control software 3.0 (Bruker Daltonics) in the reflector mode with a laser intensity of 6,000 V and an average of 2,000 shots per spectrum. The mass spectra were calibrated with external standards with masses ranging from 757.399 to 3,147.4710 m/z. Tryptsin autolysis peaks were used for internal calibration, and they are as follows: trypsin (108–115 m/z; 842.509400, trypsin (98–107 m/z; 1,045.563700), trypsin (58–77 m/z; 2,211.104000, trypsin (78–97 m/z; 2,283.180200), trypsin (58–72 m/z; 1,713.804000), trypsin (62–77 m/z; 1,774.897500), and trypsin (58–76 m/z; 2,083.009600). A range of two to 10 precursor ions were selected for MS/MS analysis. Monoisotopic mass was attributed using Bruker’s Sophisticated Numerical Annotation Procedure by collecting data from an average of 5,000 laser shots at a threshold intensity of 1,500.000 shots. The MS/MS mass spectrometry and MS/MS analyses were generated using Flex Analysis software 3.0 (Bruker Daltonics). Proteins were identified using MASCOT version 2.4.01 (http://www.matrixscience.com; Matrix Science) with the help of Biotools software (Bruker Daltonics). The following parameters were set for searches against target databases such as National Center for Biotechnology Information nonredundant (downloaded on October 1, 2012: 20,543,454 sequences, 7,050,788,919 residues), SwissProt (downloaded on October 1, 2012: 537,505 sequences, 190,795,142 residues), and Plant EST (downloaded on October 1, 2012: 157,998,786 sequences, 27,903,646,510 residues): (1) trypptic cleavage with a maximum of two missed cleavages; (2) peptide mass tolerance of 0.1 to 1.0 D; (3) peptide mass tolerance of 0.1 to 1.0 ppm; (4) a minimum of two peptides matching the protein; (5) fixed and variable modifications like carbamidomethylation of Cys and Met oxidation, respectively. In addition, we also searched in SGN (offline from Mascot) at the link ftp://ftp.solgenomics.net/genermes/Solanum_lycopersicum/annotation/ITAG2.3_release/ITAG2.3_proteins.fasta. The false discovery rate for the statistically significant proteins was found to be 5% using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995; Efroni 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 the protein expression profile was generated.

RNA Isolation and Quantitative Real-Time PCR

RNA was isolated from the outer pericarp of wild-type and hyf fruits at three ripening stages, MG, BR, and RR (each with three biological replicates), using TRI reagent (Sigma-Aldrich; TR9244) according to the manufacturer’s protocol. The isolated RNA was treated with RQI RNase Free DNase (Promega) according to the manufacturer’s recommendations to remove DNA. Reverse transcription was performed with 2 μg of RNA using the SuperScript III RT First Strand Synthesis Kit (Invitrogen) according to the manufacturer’s protocol. Quantitative real-time PCR was performed with about 4 ng of total RNA in a 10-μL reaction volume using SYBR Green PCR Master Mix (Takara) on a 7300 Fast Real Time PCR System (Applied Biosystems; http://www.appliedbiosystems.com/). Gene-specific primers were designed from the sequences obtained from the SGN database using Primer 3 software and are represented in Supplemental Table S3. The relative fold differences for each sample were determined by normalizing cycle threshold (Ct) values of each gene to the mean expression of both β-Actin and Ubiquitin genes and were calibrated using the equation 2^−ΔΔCt, which gives the relative expression of each gene. Mean ± SE (Sigmamplot version 11.0) cycle threshold values obtained from three biological replicates were used for the 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 μL Tris, pH 8.0, 150 mM NaCl, 2.5 μL of protease inhibitor cocktail per 1 mL of extraction buffer [Sigma], 1 μL PMSE, 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 the method of Bradford (1976). After 12% SDS-PAGE (Laemmli, 1970), proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore; Towbin et al., 1979). The blots were incubated with a 1:2,500 dilution of antibodies raised against bell pepper (Capsicum annuum) PAP protein followed by anti-rabbit IgG alkaline phosphatase conjugate (1:80,000 dilution; Sigma-Aldrich). The blots were finally developed using chromogenic substrate for alkaline phosphatase, 66 μL of nitroblue tetrazolium (50 mg mL−1) and 33 μL of 5-bromo-4-chloro-3-indolyl phosphate (50 mg mL−1), in 10 mL of buffer (100 mM Tris-HCl, 100 mM NaCl, and 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 h, 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 described previously (Santisree et al., 2011). For every sample, ethylene in the head-space 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 analyzed by HPLC (Shimadzu) on a C-18 column (250 × 4.6 mm, 5 μm; Phenomenex) using a 60-min isocratic gradient of methanol:acetonitrile (90:10, v/v) plus triethylamine (9 μL) at an amplitude of 23% with a 3-min interval between sonications (Sonics and Porter, Danbury, CT). Chlorophylls were distributed equally into eight 50-mL tubes and then centrifuged for 10 min at 1,200g. The plastoglobule fraction thus obtained was dialyzed against 1 mL of extraction buffer (100 mM Tris-HCl, 100 mM NaCl, and 5 mM MgCl2, pH 9.5; Sreelakshmi, 2000).

Isolation of Plastoglobules and Western Blotting

Plastoglobules were isolated from tomato fruits at the 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 homogenization buffer (450 mM sorbitol, 20 mM Tricine-KOH, pH 8.4, 10 mM EDTA, 10 mM NaHCO3, 5 mM sodium-ascorbate, 0.05% [w/v] bovine serum albumin fraction V, and 1 mM PMSE). The homogenate was filtered immediately through four layers of 20-μm Miracloth, and the filtrate was distributed equally into eight 50-mL tubes and then centrifuged for 10 min at 1,200g. Each chromoplast membrane pellet was gently resuspended in 4 to 5 mL of 0.6 × Suc/1× TrE (10× stock solution has 50 mM Tricine–KOH, pH 7.5, 2 mM EDTA, and 2 mM DTT), and these were pooled into a 50-mL Falcon tube. The membrane fraction was then sonicated five times for 40 s each on ice at an amplitude of 23% with a 3-min interval between sonications (Sonics and Materials; Vibracell VCX 1300F). The samples were disubribed in 5–6 mL aliquots into the required number of UltraClear SW28 tubes and then carefully overlaid with the Suc/1× TrE solutions in the following order: 6 mL of 38% (w/v) Suc, 6 mL of 20% (w/v) Suc, 4 mL of 15% (w/v) Suc, and finally, 8 mL of 5% (w/v) Suc to the top of the tube. These gradients were centrifuged for 16 h in a Beckman Coulter Optima L-90K centrifuge at 100,000g. The plastoglobule-rich fraction was then collected from the top layer.

The plastoglobule fraction thus obtained was dialyzed against 1× TrE buffer using a 3500 MWCO membrane (Thermo Scientific), and the protein was precipitated from the plastoglobule fraction using methanol-chloroform as essentially described by Besagni et al. (2011). After precipitation, the protein was resuspended in sample buffer, boiled, and then separated by 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) coupled to an inverted microscope (with non-descanned detector multiphoton laser). Freshly isolated plastoglobules were placed on a glass slide and covered with a coverslip. The carotenoid autofluorescence from the isolated plastoglobules was measured by exciting the emission between 500 and 510 nm (green), and chlorophyll emission was measured between 740 and 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 follows. 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 12,000g for 5 min, and the supernatant was dried in a SpeedVac. After drying, the sample was dissolved in 6 μL of n-hexane, and the absorption spectra for 1.5 μL were recorded three times in a Nanodrop spectrophotometer (ND1000; Thermo Scientific) from 350 to 700 nm.

The following genes (with accession numbers) were examined in this study: DXS, Solyc10g076920.2.1; ID1, Solyc06g055760.2.1; GGD25, Solyc04g097960.1.1; PSLY1, Solyc03g031860.2.1; PSY2, Solyc02g088130.2.1; PDS, Solyc03g123760.2.1; ZIS, AK326152.1; CRTISO, Solyc10g081650.1.1; ZDS, Solyc03g097810.2.1; LCYB1, Solyc04g040700.1.1; LCYB2, Solyc01g079480.1.1; CYCB, Solyc02g047240.1.1; CYCE, Solyc12g009860.1.1; CRTRB2, Solyc03g007960.2.1; ZEP, Solyc02g090890.2.1; VDE, Solyc06g059030.2.1; NEC1DI, Solyc07g066701.1; CYP97C29, Solyc09g051900.2.1; CYP97C1, Solyc03g083790.1.1; CRTRB1, Solyc06g092960.2.1; β-ACTIN, FJ323251.1; UBQ11, Solyc02g081170.2.1.

Supplemental Data

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

Supplemental Figure S1. Experimental design for proteomic study.

Supplemental Figure S2. Visualization of the outer pericarp in tomato fruits.

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

Supplemental Figure S4. Schematic representation of metabolic pathways and abundance of proteins identified in wild-type and mutant fruits.

Supplemental Figure S5. Schematic representation of the electron transport chain operating in the thylakoid membranes of chloroplasts.

Supplemental Figure S6. Hypothetical model representing the induction of heat-shock proteins during fruit ripening.

Supplemental Figure S7. Comparison of protein, transcript, and metabolite levels in wild-type and 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 wild-type and hp1 fruits by MALDI-TOF/TOF analysis.

Supplemental Table S2. The differentially expressed proteins (Supplemental Table S1) were compared with other studies to identify similarities and differences in the proteome complement.

Supplemental Table S3. Primers used in this study for quantitative real-time PCR analysis.

Acknowledgments

We thank Dr. Mireille Faurobert and Dr. Mark Lohse for helpful discussions. We also thank Dr. Guy Houle for his generous gift of bell pepper PAP antibody. We thank Ms. Monica Kannan for her help with MALDI. We thank Ms. Nalini Manthapuram for her help with confocal microscopy. We thank the Tomato Genetics Resource Centre for providing tomato AC, hp1, hp2, hp3, hp5, and nor seeds and the Indian Institute of Horticultural Research for tomato ‘Arka vikas’ seeds. We are also thankful to the Department of Biotechnology, Government of India Centre for Teaching and Research in Biology and Bio-technology, the Department of Science and Technology, Government of India Centre for Teaching and Research in Biology and Bio-technology, the Department of Science and Technology, Government of India Centre for Innovation and Technology, the University Grants Commission, and the New Delhi Centre for Advanced Studies for providing necessary equipment support for this work.

Received December 3, 2012; accepted February 9, 2013; published February 11, 2013.

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Integrative transcript and metabolite analysis of nutritionally enhanced DE-ETIOLATED1 downregulated tomato fruit. Plant Cell 22: 1190–1215


