Transcriptional Profiling of high pigment-2dg Tomato Mutant Links Early Fruit Plastid Biogenesis with Its Overproduction of Phytonutrients

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Phenotypes of the tomato (Solanum lycopersicum) high pigment-2dg (hp-2dg) and hp-2l mutants are caused by lesions in the gene encoding DETIOLATED1, a negative regulator of light signaling. Homozygous hp-2dg and hp-2l plants display a plethora of distinctive developmental and metabolic phenotypes in comparison to their normal isogenic counterparts. These mutants are, however, best known for the increased levels of carotenoids, primarily lycopene, and other plastid-accumulating functional metabolites. In this study we analyzed the transcriptional alterations in mature-green, breaker, and early red fruits of hp-2dg/hp-2dg plants in relation to their normal counterparts using microarray technology. Results show that a large portion of the genes that are affected by hp-2dg mutation display a tendency for up- rather than down-regulation. Ontology assignment of these differentially regulated transcripts revealed a consistent up-regulation of transcripts related to chloroplast biogenesis and photosynthesis in hp-2dg mutants throughout fruit ripening. A tendency of up-regulation was also observed in structural genes involved in phytonutrient biosynthesis. However, this up-regulation was not as consistent, positioning plastid biogenesis as an important determinant of phytonutrient overproduction in hp-2dg and possibly other hp mutant fruits. Microscopic observations revealed a highly significant increase in chloroplast size and number in pericarp cells of mature-green hp-2dg/hp-2dg and hp-2l/hp-2l fruits in comparison to their normal counterparts. This increase could be observed from early stages of fruit development. Therefore, the molecular trigger that drives phytonutrient overproduction in hp-2dg and hp-2l mutant fruits should be initially traced at these early stages.

Enhanced pigmentation is of a major economic importance in fruits and vegetables, contributing to their visual and functional properties. Five photomorphogenic high pigment (hp) mutations have been identified among tomato (Solanum lycopersicum) accessions hp-1, hp-10, hp-2, hp-2l, and hp-2dg that map to two genes: HP-1 and HP-2. The origins of these mutations have been lately extensively summarized (Lieberman et al., 2004; Levin et al., 2006). Similar to the other hp mutants, hp-2dg/hp-2dg plants are characterized by exaggerated light responsiveness, dark-green foliage, and intense fruit pigmentation (Mustilli et al., 1999; Levin et al., 2003; Lieberman et al., 2004). Tomato plants carrying the hp-2dg mutation are considered to be superior over most other known hp mutations because they usually produce tomato processing hybrids with higher and reproducible pigmentation, and plants with better horticultural performances (Levin et al., 2003).

The higher fruit pigmentation characterizing hp-2dg plants is due to elevated levels of chlorophylls in immature fruits and of carotenoids, primarily lycopene, throughout fruit ripening from very early breaker to red-ripe stages (Mochizuki and Kamimura, 1984; Wann et al., 1985; Peters et al., 1989; Mustilli et al., 1999; Levin et al., 2003; Bino et al., 2005). Fruits of hp-2dg mutants also display, as early as mature-green stage and onwards, increased levels of other functional metabolites such as vitamins (C and E) and several flavonoids (Mochizuki and Kamimura, 1984; Wann et al., 1985; Bino et al., 2005). Such characteristics render the hp-2dg genotype a candidate for nongenetically modified functional tomatoes (Levin et al., 2003; Bino et al., 2005) and demonstrate a conceptual link between light signaling and overproduction of fruit functional metabolites in tomato fruits (Bino et al., 2005).

The HP-2 gene was cloned and found to encode the tomato homolog of the Arabidopsis (Arabidopsis thaliana) nuclear protein DETIOLATED1 (DET1), a central negative regulator of photomorphogenesis (Chory, 1993; Pepper et al., 1994; Mustilli et al., 1999). Microarray analyses carried out using viable det1 alleles of Arabidopsis displayed vast transcriptional alterations in the mutant seedlings compared to their isogenic normal counterparts. These alterations include transcriptional induction of light-regulated genes as well as genes associated with plastid biogenesis in dark-grown det1 plants. Also, in light-grown det1 plants of tomato seedlings, a large portion of differentially regulated transcripts are affected by hp-2dg mutation display a tendency for up-regulation rather than down-regulation. Ontology assignment of these differentially regulated transcripts reveals a consistent up-regulation of transcripts related to chloroplast biogenesis and photosynthesis in hp-2dg mutants throughout fruit ripening. A tendency of up-regulation was also observed in structural genes involved in phytonutrient biosynthesis. However, this up-regulation was not as consistent, positioning plastid biogenesis as an important determinant of phytonutrient overproduction in hp-2dg and possibly other hp mutant fruits. Microscopic observations revealed a highly significant increase in chloroplast size and number in pericarp cells of mature-green hp-2dg/hp-2dg and hp-2l/hp-2l fruits in comparison to their normal counterparts. This increase could be observed from early stages of fruit development. Therefore, the molecular trigger that drives phytonutrient overproduction in hp-2dg and hp-2l mutant fruits should be initially traced at these early stages.

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both Arabidopsis and tomato, chloroplast development was reported in plant tissues that normally lack chloroplasts (Chory and Peto, 1990; Mustilli et al., 1999; Schroeder et al., 2002). Arabidopsis mutants with lethal det1 alleles show transcriptional expression profiles similar to those normally observed in seedlings grown under high light intensities, usually characterizing light stress (Ma et al., 2003). In addition, transcriptional induction of several genes frequently up-regulated by pathogen-plant interactions was reported in healthy Arabidopsis det1 mutant seedlings (Mayer et al., 1996).

Three light-regulated genes, CHLOROPHYLL a/b-BINDING PROTEIN, RUBISCO SMALL SUBUNIT, and CHALCONE SYNTHASE, were demonstrated to be transcriptionally up-regulated in the hp-1 mutant plants of tomato compared to their semi-isogenic normal controls (Peters et al., 1998). Increased plastid number and compartment size in leaf and fruit cells were reported as major phenotypic traits of hp-1 mutant plants (Cookson et al., 2003). The recent cloning of the HP-1 gene revealed that it encodes the tomato homolog of the UV-DAMAGED DNA BINDING PROTEIN1 (DDB1; Lieberman et al., 2004; Liu et al., 2004), known to contribute to the initial UV damage response by stimulating nucleotide excision repair in human cells (Wakasugi et al., 2002). It was also recently found that DET1 and DDB1 interact biochemically and genetically to suppress photomorphogenesis in Arabidopsis (Schroeder et al., 2002; Liu et al., 2004) and to enhance the activity of ubiquitin-conjugating enzymes in both Arabidopsis (Yanagawa et al., 2004) and humans (Wertz et al., 2004). These findings can explain the significant phenotypic similarities that exist between the different tomato hp mutants (Lieberman et al., 2004).

Ripe-red fruits of the various hp tomato mutants share strong metabolic similarities. Earlier reports describing levels of metabolites in hp tomato mutants are limited and focus primarily on the increased levels of fruit carotenoids (lycopene and carotenes) observed in ripe-red fruits (Mochizuki and Kamimura, 1984; Wann et al., 1985). Interestingly, however, mature fruits of plants carrying the hp-1 mutation were also found to contain elevated levels of the flavonoid quercetin (Yen et al., 1997) and of ascorbic acid (vitamin C; Mochizuki and Kamimura, 1984). A study carried out by our group identified similar increases in quercetin levels in the fruit peel of the tomato hp-2 and hp-22 mutants (Levin et al., 2006). In another study, targeted metabolite analyses as well as large-scale nontargeted metabolite-profiling methods were used to identify differences in metabolite composition between fruits of the hp-26 mutant and its isogenic control. The results show that hp-26 fruits are characterized by overproduction of many plastid-accumulating metabolites, several of which are known for their strong antioxidan or photoprotective properties and may be implicated as resources recruited by the hp-26 mutant plants in response to light stress (Bino et al., 2005). Based on these metabolomic results, it would be reasonable to hypothesize that similar large-scale overexpression would be expected in genes that are either related to metabolic pathways and/or such that are related to the response of plants to light cues. To validate this hypothesis we have carried out a comprehensive transcriptional profiling of fruits obtained from hp-26 mutant plants in comparison to their semi-isogenic normal counterparts. Further, analysis of global gene expression in the developing fruit of hp mutants can be an effective approach to dissect the molecular mechanism by which hp mutants overproduce phytonutrients. Such dissection may be useful in developing future strategies to manipulate levels of carotenoids and possibly other secondary metabolites in tomato fruits and possibly fleshy fruit of other species.

RESULTS

General Considerations

This study was designed to analyze the transcriptional alterations in fruits harvested from tomato det1 mutant plants (hp-26/hp-26) in relation to their normal isogenic counterparts using microarray technology. This study complements a former metabolomic-profiling analysis showing that both targeted and nontargeted metabolites are significantly increased in hp-26/hp-26 mutant fruits (Bino et al., 2005). We hypothesized that analysis of global gene expression can be useful in elucidating the molecular mechanism by which hp tomato mutants overproduce fruit phytonutrients.

Validation of Microarray Results

To validate the microarray expression results, 11 annotated genes displaying a significant difference in expression in at least one ripening stage were selected for quantitative reverse transcription (qRT)-PCR analysis. qRT-PCR reactions were carried out on c-DNA synthesized from the original RNA samples prior to mRNA amplification. Results obtained from the TOM1 microarray and the qRT-PCR reactions were transformed to their natural logarithm prior to the linear regression analysis presented in Figure 1. This analysis shows a highly significant fit between the microarray results and the qRT-PCR results, 10^{-9} < P(b) < 10^{-5}, with R^2 = 0.7032, equivalent to a linear correlation of approximately 0.84.

Differences measured by qRT-PCR appear greater than those detected by microarray hybridization (Fig. 1). We usually observe this tendency while analyzing the association between qRT-PCR and microarray values, suggesting that qRT-PCR is more discriminative. On the other hand, the average effect values obtained by qRT-PCR tend to share higher error rates, leading to very similar statistical significance outcome. For instance, the average fold difference of the microarray results presented in Figure 1 is 1.88 ± 0.28 prior to the transformation, while the corresponding fold difference obtained by qRT-PCR is 3.62 ± 0.72. These values
represent a 1.92-fold increase in qRT-PCR relative to microarray results and also a higher 2.57-fold increase in the corresponding standard errors. Thus, from a statistical point of view these results are highly comparable. Because qRT-PCR tends to display higher fold differences compared to microarray, the regression line between these two variables will not usually cross the origin as displayed in Figure 1. However, the regression slope (b), the true indicator of fit between these two techniques, was found in this study to be highly significant [$10^{-9} < P(b) < 10^{-5}$] and therefore our microarray results should be considered of good quality.

**General Transcriptional Alterations Displayed by hp-2dg Mutant Fruits**

The microarray results presented herein below summarize the fold differences in the transcription obtained in fruits harvested from hp-2dg/hp-2dg plants relative to their normal counterparts. We focused on data from 4,986 cDNA spots, showing reliable signal-to-noise ratio, derived from a total of 13,340 spots immobilized on the TOM1 chip (Table I). Subsequent filtering for genes that are significantly altered was performed separately for each of the ripening stages under study. This analysis revealed that 1,928 (38.7%), 421 (8.4%), and 671 (13.5%) spots were significantly altered in hp-2dg/hp-2dg mutant fruits at the mature-green, breaker, and red stages, respectively (Table I). Further analysis of these data, to include only genes showing more than 2-fold alteration in fruits harvested from the hp-2dg/hp-2dg genotype, show the following patterns (Table I): (1) The hp-2dg genotype is characterized by a transcriptional alteration of a large number of gene transcripts, compared to normal plants at all ripening stages analyzed; (2) more gene transcripts were found up- rather than down-regulated in the hp-2dg/hp-2dg genotype at all developmental stages analyzed; and (3) more genes were transcriptionally altered at the mature-green stage compared to the other two ripening stages analyzed.

Similar results were obtained analyzing annotated genes (Table I). Among the 4,986 reliable cDNA spots identified, 1,815 were found to represent annotated genes. These genes follow very similar regulation patterns, calculated as percentages, outlined herein above for total cDNA spots analyzed.

Annotated cDNA spots showing reliable signal-to-noise ratio and a significant more than 2-fold transcriptional alteration (Table I) were sorted according to fruit ripening stage and their biological function. Up- and down-regulated genes are presented in Supplemental Figures S1 and S2, respectively. Of the 149, 86, and 116 annotated cDNA spots displaying a significant more than 2-fold up-regulation at the mature-green, breaker, and red fruits, respectively (Table I), 47 were found constitutively up-regulated (Supplemental Fig. S1). These 47 genes comprise 31.5%, 54.6%, and

### Table 1. General characterization of the transcriptional alterations in pericarp tissue of hp-2dg/hp-2dg tomato mutant fruits in three developmental stages based on total cDNA spots and cDNA spots representing annotated genes

<table>
<thead>
<tr>
<th>Basis of Calculation</th>
<th>Ripening Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mature Green</td>
</tr>
<tr>
<td>Total cDNA spots</td>
<td>4,986</td>
</tr>
<tr>
<td>Statistically significant [P(b) &lt; 0.05]</td>
<td>1,928 (38.7%)</td>
</tr>
<tr>
<td>Up-regulated &gt;2-fold</td>
<td>387 (7.8%)</td>
</tr>
<tr>
<td>Down-regulated &gt;2-fold</td>
<td>153 (3.1%)</td>
</tr>
<tr>
<td>Total altered genes (&gt;2-fold)</td>
<td>540 (10.8%)</td>
</tr>
<tr>
<td>cDNA spots representing annotated genes</td>
<td></td>
</tr>
<tr>
<td>Total annotated unique genes analyzed</td>
<td>1,815</td>
</tr>
<tr>
<td>Statistically significant [P(b) &lt; 0.05]</td>
<td>747 (41.2%)</td>
</tr>
<tr>
<td>Up-regulated &gt;2-fold</td>
<td>149 (8.2%)</td>
</tr>
<tr>
<td>Down-regulated &gt;2-fold</td>
<td>69 (3.8%)</td>
</tr>
<tr>
<td>Total altered genes (&gt;2-fold)</td>
<td>218 (12.0%)</td>
</tr>
</tbody>
</table>
40.5% of the genes found to be >2-fold up-regulated at mature-green, breaker, and red fruits, respectively. These results are in sharp contrast to the single gene that was found constitutively down-regulated (Supplemental Fig. S2), demonstrating again the transcriptional activation of genes in the hp-2dg mutant fruits.

We have further sorted the 47 genes demonstrating a constitutive up-regulation according to their biological function, taking into account only genes that are fully annotated. These genes were categorized into 10 distinct functional groups (Supplemental Fig. S1). Although each of these functional groups may represent an important physiological, biochemical, or developmental property of the pleotropic hp-2dg mutant, we will in particular discuss herein below genes related to carotenoid biosynthetic pathway, plastid biogenesis, photosynthesis, and stress response.

### Transcriptional Alterations of Carotenogenesis Genes in hp-2dg Mutant Fruits

Ripe-red fruits of hp mutants are best known for their capacity to overproduce carotenoids, primarily lycopene (Bino et al., 2005; Table II). We have therefore focused initially on structural genes of the plastid methlyerythritol phosphate (MEP) pathway, leading to carotenoid biosynthesis. The TOM1 microarray chip harbors an incomplete set of genes of this pathway, with a slight overrepresentation of genes operating at its early steps. Nonetheless, previous studies suggested that the reactions catalyzed by DEOXYXYLULOSE 5-PHOSPHATE SYNTHASE (DXS), PHYTOENE SYNTHASE (PSY), and HYDROXYMETHYBUTENYL DI-PHOSPHATE REDUCTASE (HDR) exert the greatest control over the production of plastid isoprenoids and carotenoids during tomato fruit ripening (Lois et al., 2000; Fraser et al., 2002; Botella-Pavia, et al., 2004). DXS and PSY genes are represented on the TOM1 slide while HDR is not. We have therefore analyzed the expression rate of HDR and three additional carotenogenesis genes encoding ζ-CAROTENE DESATURASE (ZDS), chromoplast-specific form of GERANYLGERANYL DI-PHOSPHATE REDUCTASE (GDS), and LYCOPENE β-CYCLASE by qRT-PCR. Results, presented in Table III, show the following properties: (1) At all ripening stages, more genes were found up- rather than down-regulated. Only three such genes show a tendency of transcriptional down-regulation at a relatively low significance level [0.01 < P(t) < 0.05]. (2) Two structural genes of the carotenoid biosynthetic pathway were found constitutively up-regulated at all three ripening stages examined: GERANYL DIPHOSPHATE SYNTHASE (GDS; SGN-U145376) and PHYTOENE DESATURASE (SGN-U150737). (3) The highest up-regulation was observed for GAPS at the breaker stage and for ZDS at the red stage. (4) Only three genes showed transcriptional up-regulation at the highest significance level examined [P(t) < 0.001]. All three genes displayed such highly significant transcriptional alteration at the mature-green stage and only two of them also displayed more than 2-fold increase in transcription: GDS and 1-DEOXY-D-XYLULOSE-5-PHOSPHATE REDUCTOISOMERASE.

Taken together, these results indicate inconsistent up-regulation of the MEP pathway, including carotenogenesis genes in hp-2dg mutant fruits, particularly when analyzing the results under stringent significance levels. Similar results were obtained when analyzing the transcriptional profile of structural genes of the flavonoid and vitamin (C and E) biosynthetic pathways, metabolites known to accumulate significantly in hp-2dg mutant fruits (data not shown).

### Major Transcriptional Effects of the hp-2dg Mutation

Among the 1,815 annotated unique genes analyzed, 121 were found to be related to chloroplast biogenesis or photosynthesis, representing 6.7%. Throughout all developmental stages tested in this study, gene transcripts related to chloroplast biogenesis or photosynthesis displayed a strong and consistent up-regulation in hp-2dg mutant fruits and comprised 36.2%, 47.3%, and 37.7% of the up-regulated (2-fold or more) and fully annotated unique genes in mature-green, breaker, and red fruits, respectively. These results are in sharp contrast to the single gene that was found constitutively down-regulated (Supplemental Fig. S2), demonstrating again the transcriptional activation of genes in the hp-2dg mutant fruits.

### Table II. Carotenoid profile in ripe-red tomato pericarp tissue of two det1 mutant genotypes, hp-2dg and hp-2j, in comparison to their isogenic normal counterparts

Values represent average carotenoid concentrations (in μg g⁻¹ fresh weight) ± s. Different superscript letters indicate statistically significant differences between means of each carotenoid within each genotype pair (P(F) < 0.005).

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Genotype</th>
<th>hp-2dg/hp-2j</th>
<th>(+/−)</th>
<th>hp-2j/hp-2</th>
<th>(+/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene</td>
<td>6.53 ± 0.17</td>
<td>5.64 ± 0.17</td>
<td>7.60 ± 0.52</td>
<td>3.41 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>Phytofluene</td>
<td>0.27 ± 0.07</td>
<td>0.38 ± 0.02</td>
<td>1.08 ± 0.18</td>
<td>0.15 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>ζ-Carotene</td>
<td>0.92 ± 0.02</td>
<td>0.38 ± 0.07</td>
<td>0.76 ± 0.14</td>
<td>0.25 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Lutein</td>
<td>2.09 ± 0.25</td>
<td>0.12 ± 0.03</td>
<td>2.27 ± 0.12</td>
<td>0.57 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Lycopene</td>
<td>0.81 ± 0.04</td>
<td>1.64 ± 0.07</td>
<td>1.02 ± 0.09</td>
<td>1.88 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>β-Carotene</td>
<td>169.02 ± 6.13</td>
<td>76.70 ± 3.94</td>
<td>245.60 ± 10.77</td>
<td>88.81 ± 6.25</td>
<td></td>
</tr>
<tr>
<td>Total carotenoids</td>
<td>200.95 ± 6.12</td>
<td>86.90 ± 4.06</td>
<td>317.63 ± 9.81</td>
<td>100.20 ± 6.77</td>
<td></td>
</tr>
</tbody>
</table>
Table III. Fold changes in transcription of the MEP pathway, including carotenogenesis genes, observed in fruits of hp-2dg/hp-2dg mutant plants relative to their normal counterparts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ripening Stage</th>
<th>MG</th>
<th>B</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXS</td>
<td></td>
<td>0.8^a</td>
<td>0.6^a</td>
<td>0.7^ns</td>
</tr>
<tr>
<td>1-DEOXY-d-XYLULOSE-5-PHOSPHATE REDUCTOISOMERASE</td>
<td></td>
<td>2.1^b</td>
<td>0.8^a</td>
<td>0.7^ns</td>
</tr>
<tr>
<td>2-C-METHYL-d-ERYTHRITOL 2,4-CYCLODIPHOSPHATE SYNTHASE</td>
<td></td>
<td>0.7^ns</td>
<td>1.0^a</td>
<td>1.3^ns</td>
</tr>
<tr>
<td>HYDROXYMETHYLBUTENYL DIPHOSPHATE SYNTHASE</td>
<td></td>
<td>1.6^b</td>
<td>1.0^a</td>
<td>1.4^b</td>
</tr>
<tr>
<td>HDR</td>
<td></td>
<td>1.0^ns</td>
<td>1.4^b</td>
<td>2.4^b</td>
</tr>
<tr>
<td>ISOPENTENYL DIPHOSPHATE ISOMERASE1</td>
<td></td>
<td>0.8^a</td>
<td>1.4^b</td>
<td>1.2^ns</td>
</tr>
<tr>
<td>GDS</td>
<td></td>
<td>2.9^a</td>
<td>1.4^b</td>
<td>1.6^b</td>
</tr>
<tr>
<td>GGP5</td>
<td></td>
<td>1.1^ns</td>
<td>4.9^b</td>
<td>1.4^c</td>
</tr>
<tr>
<td>GGP5 (chromoplast specific)</td>
<td></td>
<td>1.0^ns</td>
<td>0.6^ns</td>
<td>1.9^ns</td>
</tr>
<tr>
<td>PSY1</td>
<td></td>
<td>1.7^b</td>
<td>1.0^a</td>
<td>1.5^b</td>
</tr>
<tr>
<td>PHYTOENE DESATURASE</td>
<td></td>
<td>1.6^a</td>
<td>1.3^b</td>
<td>1.3^c</td>
</tr>
<tr>
<td>ζ-CAROTENE DESATURASE</td>
<td></td>
<td>0.9^a</td>
<td>1.1^a</td>
<td>3.9^b</td>
</tr>
<tr>
<td>Lycopene β-CYCLASE</td>
<td></td>
<td>1.8^b</td>
<td>1.0^a</td>
<td>0.9^ns</td>
</tr>
<tr>
<td>Lycopene β-CYCLASE (chromoplast specific)</td>
<td></td>
<td>0.2^a</td>
<td>0.6^a</td>
<td>1.8^a</td>
</tr>
<tr>
<td>β-CAROTENE HYDROXYLASE</td>
<td></td>
<td>0.8^a</td>
<td>1.0^ns</td>
<td>0.6^c</td>
</tr>
</tbody>
</table>

Accumulation of Chloroplasts Begins at Early Development of Mutant hp-2 Fruits

To further dissect the increase in chloroplast numbers in the hp-2^dg^ mutant fruits and to monitor the developmental time point of plastid proliferation increase, we have carried out a detailed confocal microscopy study focusing on the very early stages of fruit development in hp-2^dg^ mutant plants and their normal counterparts. Our results show that the increase in plastid number observed in hp-2^dg^ plants is an early developmental event (Fig. 3; Table VIII). Similar results were obtained analyzing developing fruits of hp-2 tomato plants, allelic to hp-2^dg^, and their semi-isogenic controls (Table VIII).

DISCUSSION

Fruits and vegetables constitute a major component of our diet, providing fiber, vitamins, minerals, and many other phytonutrients that promote, or at least maintain good health (Willcox et al., 2003; Fraser and Bramley, 2004). Fleshy fruits, such as tomatoes, contain high levels of several of these ingredients. Nonetheless, efforts have been invested in increasing the content and diversifying phytonutrients, such as carotenoids and flavonoids, in the tomato fruit (Verhoeven et al., 2002; Fraser and Bramley, 2004; Levin et al., 2004). Strategies to achieve this task focus mainly on transgenic approaches (Fray et al., 1995;...
Table V. Fold changes in transcription of genes related to photosynthesis in hp-2dg/hp-2dg mutant plants relative to their normal counterparts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fruit Developmental Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MG</td>
</tr>
<tr>
<td>CHLOROPHYLL a/b-BINDING PROTEIN1B</td>
<td>3.2a</td>
</tr>
<tr>
<td>CHLOROPHYLL a/b-BINDING PROTEIN4</td>
<td>1.9a</td>
</tr>
<tr>
<td>CHLOROPHYLL a/b-BINDING PROTEIN3C</td>
<td>1.0ns</td>
</tr>
<tr>
<td>CHLOROPHYLL a OXYGENASE</td>
<td>6.3a</td>
</tr>
<tr>
<td>(CHLOROPHYLL b SYNTHASE)</td>
<td></td>
</tr>
<tr>
<td>CYTOCHROME B6-F COMPLEX</td>
<td>3.2a</td>
</tr>
<tr>
<td>IRON-SULFUR SUBUNIT2</td>
<td></td>
</tr>
<tr>
<td>FERREDOXIN—NADP REDUCTASE</td>
<td>3.6a</td>
</tr>
<tr>
<td>OXYGEN-EVOLVING ENHANCER PROTEIN2</td>
<td>3.1a</td>
</tr>
<tr>
<td>PHOSPHORIBULOKINASE PRECURSOR</td>
<td>4.6a</td>
</tr>
<tr>
<td>PSII 22-kD PROTEIN</td>
<td>5.8a</td>
</tr>
<tr>
<td>Plasticid aldolase NPA1D1</td>
<td>2.6a</td>
</tr>
<tr>
<td>PLASTOCYANIN</td>
<td>3.6a</td>
</tr>
<tr>
<td>RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN2A CARBOXYLASE SMALL CHAIN3A/3C</td>
<td>3.6a</td>
</tr>
<tr>
<td>RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN3A/3C</td>
<td>10.2a</td>
</tr>
<tr>
<td>RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN3A/3C</td>
<td>3.9a</td>
</tr>
<tr>
<td>RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN1</td>
<td>2.5a</td>
</tr>
<tr>
<td>RIBULOSE BISPHOSPHATE CARBOXYLASE/OXGENASE ACTIVASE</td>
<td></td>
</tr>
<tr>
<td>SEDOHEPTULOSE-1,7-BISPHOSPHATASE</td>
<td>3.2a</td>
</tr>
</tbody>
</table>

Ripening stages: MG, Mature green; B, breaker; R, red. Statistical significance: *ns, Statistically insignificant; *t, P(t) < 0.001; *t, 0.01 ≤ P(t) < 0.05.

Table VI. Fold changes in transcription of genes related to light stress and ROS scavenging in fruits of hp-2dg/hp-2dg mutant plants relative to their normal counterparts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Ripening Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MG</td>
</tr>
<tr>
<td>ASCORBATE PEROXIDASE</td>
<td>3.1a</td>
</tr>
<tr>
<td>CATALASE</td>
<td>6.5a</td>
</tr>
<tr>
<td>CHAPERONIN21</td>
<td>2.5a</td>
</tr>
<tr>
<td>ELIP</td>
<td>9.1a</td>
</tr>
<tr>
<td>FERREDOXIN-THIOREDOXIN</td>
<td>2.1a</td>
</tr>
<tr>
<td>REDUCTASE</td>
<td></td>
</tr>
<tr>
<td>Fish METALLOPROTEASE</td>
<td>2.9a</td>
</tr>
<tr>
<td>GLUTATHIONE PEROXIDASE</td>
<td>2.0b</td>
</tr>
<tr>
<td>GLUTATHIONE S-TRANSFERASE</td>
<td>3.3b</td>
</tr>
</tbody>
</table>

Ripening stages: MG, Mature green; B, breaker; R, red. Statistical significance: *ns, Statistically insignificant; *t, P(t) < 0.001; *t, 0.01 ≤ P(t) < 0.05.
very well with a previous metabolite-profiling study carried out on hp-2\(^{dg}\) mutant plants, showing that fruits harvested from these plants are characterized by overproduction of many metabolites (Bino et al., 2005). In this study (Bino et al., 2005), both targeted and nontargeted metabolite analyses usually displayed overproduction of metabolites in mature-green, breaker, and red fruits harvested from these mutant plants. Noteworthy is the strong up-regulation of mass signals detected by HPLC coupled to high-resolution quadrupole time-of-flight mass spectrometry in hp-2\(^{dg}\) mutant fruits compared to their isogenic counterparts. Out of a total of 445 mass signals found to significantly differentiate between ripe-red fruits harvested from hp-2\(^{dg}\) mutant plants and their normal isogenic counterparts, 383 (approximately 86\%) mass signals displayed higher levels in hp-2\(^{dg}\) mutant plants, while only 62 (approximately 14\%) displayed lower levels. Hundreds of genes were also found to be altered in both dark- and light-grown det1 mutant Arabidopsis seedlings (Hu et al., 2002), with a tendency for over- rather than underexpression (Schroeder et al., 2002). Taken together, these results emphasize the wide transcriptional regulatory nature of DET1 both at the seedling (vegetative) and fruit (reproductive) levels. The tendency for overexpression of genes in det1 mutant plants reinforces the perception that a defective DET1 tends to remove negative regulation from light regulated and possibly other genes, usually resulting in enhanced transcription rates of these genes (Christopher and Hoffer, 1998; Schroeder et al., 2002). Notably, work with the tomato DET1 showed that it interacts with the nonacetylated tail of histone H2B and it has been proposed to modulate chromatin structure and to affect light-dependent transcriptional activities (Benvenuto et al., 2002).

Table VII. Number of chloroplasts per cell, chloroplast size and pigment content (chlorophyll and total carotenoids) in fruit pericarp tissue of hp-2\(^{dg}\) and hp-2\(^{j}\) mutant plants and their normal controls

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Chloroplast No.</th>
<th>Chloroplast Size</th>
<th>Chlorophyll</th>
<th>Total Carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu \text{m}^2)</td>
<td>(\mu \text{g g}^{-1} \text{ fresh weight})</td>
<td>(\mu \text{g g}^{-1} \text{ fresh weight})</td>
<td></td>
</tr>
<tr>
<td>(hp-2^{dg})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(hp-2^{dg}/hp-2^{dg})</td>
<td>1,709.3± 110.5 (n = 50)</td>
<td>25.5± 0.3 (n = 5,055)</td>
<td>115.0± 2.5</td>
<td></td>
</tr>
<tr>
<td>(+/+)</td>
<td>423.6± 28.5 (n = 45)</td>
<td>11.9± 0.2 (n = 4,345)</td>
<td>8.2± 1.3</td>
<td></td>
</tr>
<tr>
<td>Fold increase</td>
<td>4.0</td>
<td>2.1</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>(hp-2^{j})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(hp-2^{j}/hp-2^{j})</td>
<td>3,167.5± 274.4 (n = 33)</td>
<td>9.7± 0.34 (n = 271)</td>
<td>76.1± 3.21</td>
<td></td>
</tr>
<tr>
<td>(+/+)</td>
<td>723.4± 59.9 (n = 33)</td>
<td>6.1± 0.38 (n = 186)</td>
<td>8.7± 0.95</td>
<td></td>
</tr>
<tr>
<td>Fold increase</td>
<td>4.4</td>
<td>1.6</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>(Fold increase)</td>
<td></td>
<td></td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Visual display of the effects of hp-2\(^{dg}\) mutation on chloroplast biogenesis in mature-green fruits compared to their normal controls. Images of hp-2\(^{dg}\)/hp-2\(^{dg}\) mutant plants (A, C, E, and G) are shown relatively to their normal counterparts (B, D, F, and H). Whole fruits (A and B), pericarp tissue (C and D), single cells (E and F), and chloroplasts inside cells (G and H). Scale bars: C and D = 100 \(\mu\)m, E and F = 50 \(\mu\)m, G and H = 5 \(\mu\)m.
Interestingly, the positive transcriptional effect of the \( \text{hp}^{-2\text{dg}} \) is retained throughout fruit ripening. At the mature-green stage, approximately 68% of the annotated genes that display differential regulation show transcriptional up-regulation in \( \text{hp}^{-2\text{dg}} \) mutant compared to its normal counterpart (based on Table I). This ratio rises sharply at breaker stage (approximately 99%) and although it is reduced at the red stage (approximately 79%), it is still maintained at high levels (Table I). These results indicate that the removal of the negative regulation attributed to a functional mutation in \( \text{DET1} \) is constitutive throughout fruit ripening.

Down-regulated gene transcripts displayed by \( \text{hp}^{-2\text{dg}} \) mutants can be linked to an array of transcription factors that are affected due to tomato \( \text{det1} \) mutation. More than 2-fold alteration was detected in our study at levels of 23, nine, and 14 transcripts for various signal transduction elements and transcription factors in mature-green, breaker, and red stages, respectively (Supplemental Table S2), demonstrating a potential disconcert in the regulatory network of a \( \text{det1} \) tomato mutant during ripening.

One of the major characteristics of \( \text{hp} \) mutant plants is the significantly higher content of fruit lycopene as well as other carotenoids (Bino et al., 2005; Table II). Throughout this study we have profiled 15 genes encoding enzymes of the MEP pathway responsible for fruit carotenoid synthesis (Table III). Consistent with the previously mentioned tendency of transcriptional induction of genes in \( \text{hp}^{-2\text{dg}} \) fruits, up-regulation in transcription was usually obtained for most of these genes. However, this up-regulation was usually very moderate and not consistent throughout fruit ripening (Table III), suggesting the involvement of another mechanism for carotenoid accumulation. Noteworthily, these results are in agreement with a preliminary transcriptional-profiling experiment we carried out using RNA extracted from mature-green, breaker, and red stages, respectively (Figure 3).

![Figure 3](https://www.plantphysiol.org/figure/3)

Figure 3. Visual display of chloroplasts in pericarp tissue cells of \( \text{hp}^{-2\text{dg}}/\text{hp}^{-2\text{dg}} \) mutant fruits in comparison to their normal counterparts. Images of \( \text{hp}^{-2\text{dg}}/\text{hp}^{-2\text{dg}} \) mutant fruit cells (A, C, and E) are shown relatively to their normal counterparts (B, D, and F). Two (A and B), 8 (C and D), and 14 (E and F) DPA are displayed. Scale bars: A and B = 5 μm; C, D, E, and F = 50 μm.

| Table VIII. Chloroplast number per cell in pericarp tissue of developing fruits harvested from \( \text{hp}^{-2\text{dg}} \) and \( \text{hp}^{-2\text{j}} \) mutant plants in comparison to their normal controls |
|-------------------------------------------------|---------|---------|---------|
| Genotype                                       | DPA     |         |         |
|                                                 | 3       | 8       | 14      |
| \( \text{hp}^{-2\text{dg}} \)                  |         |         |         |
| \( \text{hp}^{-2\text{dg}}/\text{hp}^{-2\text{dg}} \) | 16.0 ± 1.12 (\( n = 44 \)) | 182.4 ± 8.36 (\( n = 32 \)) | 324.1 ± 12.5 (\( n = 25 \)) |
| \( +/+ \)                                       | 13.9 ± 0.73 (\( n = 68 \)) | 72.9 ± 5.14 (\( n = 32 \)) | 124.7 ± 9.1 (\( n = 24 \)) |
| Fold increase                                  | 1.1     | 2.5     | 2.6     |
|                                                 | 2       | 4       | 6       | 10      |
| \( \text{hp}^{-2\text{j}} \)                  |         |         |         |
| \( \text{hp}^{-2\text{j}}/\text{hp}^{-2\text{j}} \) | 10.7 ± 0.79 (\( n = 18 \)) | 14.6 ± 1.02 (\( n = 18 \)) | 18.4 ± 1.36 (\( n = 18 \)) |
| \( +/+ \)                                       | 10.6 ± 0.61 (\( n = 18 \)) | 12.2 ± 0.69 (\( n = 18 \)) | 16.4 ± 1.42 (\( n = 18 \)) |
| Fold increase                                  | 1.0     | 1.2     | 1.1     | 2.9     |
ripe-red tomato fruits harvested within the framework of our collaborative metabolomic study (Bino et al., 2005) using the Profood array. The Profood array was designed and printed in Plant Research International, Wageningen, The Netherlands and contains 1,045 70-mer oligonucleotides representing metabolic pathway-related genes from tomato (Lieberman, 2004). Our current study was designed to carry out a similar study under more stringent experimental layout (randomized blocks rather than randomized individual plants), utilizing genotypes ensuring higher reproducibility (as outlined in “Materials and Methods”), targeting fruit pericarps, the main site of plastidial phytonutrient accumulation, rather than whole fruits, and the TOM1 array representing many other genes, not only structural genes of metabolic pathways.

Previous studies displayed an association between DET1 function and chloroplast biogenesis. Notably, development of chloroplasts in tissues that normally do not harbor such organelles was detected in Arabidopsis and also tomato det1 mutants (Chory and Peto, 1990; Mustilli et al., 1999). Our results on fruit transcriptional profiles of tomato det1 mutant, hp-2dg, usually demonstrate consistent and highly significant transcriptional induction of genes related to chloroplast structure, function, and biogenesis. Messenger RNAs for plastid protein translation machinery are induced in fruit pericarp tissue of tomato hp-2dg mutant in mature-green and subsequent ripening stages (Table IV; Supplemental Figs. S3, S4, and S5). In addition, genes encoding proteins of photosynthetic apparatus show even higher transcriptional up-regulation at most fruit developmental stages tested (Table V). It is now clear that nuclear photosynthesis genes are regulated by signals from chloroplasts (Susek and Chory, 1992; Rodermel, 2001; Surpin et al., 2002).

The induction of genes related to photosynthesis and chloroplast biogenesis correlates well with the significantly increased number and size of chloroplasts in pericarp cells of mature-green hp-2dg fruit compared with their normal controls (Table VII; Fig. 2). Similar results were obtained in this study comparing fruits of hp-2 tomato mutant, allelic to hp-2dg, with its semi-isogenic control, thus establishing the effect of tomato det1 mutations on chloroplast biogenesis in tomato fruit pericarp cells (Table VII). DET1 is a part of a protein complex that contains DDB1, mutations in which cause the hp-1 and hp-1m phenotypes of tomato (Schroeder et al., 2002; Lieberman et al., 2004; Liu et al., 2004). Interestingly, the tomato hp-1 genotype was also reported to have increased plastome DNA concentration and plastid number in leaves and fruits (Yen et al., 1997; Cookson et al., 2003), underlining also a functional link between DDB1 and DET1 with respect to plastid biogenesis.

Total chlorophyll, accumulating in chloroplasts, and total carotenoids, accumulating in chloroplasts, increased 14- and 2.3-fold, respectively, in fruit pericarp tissue of hp-2dg compared to its normal controls. These increases cannot be directly explained by the mostly moderate up-regulation of transcripts encoding enzymes for chlorophyll synthesis (less than 2-fold, data not shown) and carotenogenic genes (Table III). On the other hand, microscopic observations carried out in this study displayed 4-fold higher chloroplast number per cell and 2.1-fold larger chloroplast area in hp-2dg fruit pericarp cells, suggesting 8.4-fold cumulative increase of plastid compartment size (Table VII). It may therefore be concluded that out of these two determinants, i.e. plastid biogenesis and transcriptional activation of structural biosynthetic genes, the estimated 8.4-fold increase in chloroplast compartment size is more important in providing the high vitamin and pigment content in hp-2dg fruits. Interestingly, a similar approximately 7-fold increase in plastid compartment size could be calculated in hp-2, an allele of hp-2dg characterized by 8.7- and 3.2-fold increase in chlorophyll and total carotenoid content, respectively (Table VII).

Interestingly, pericarp cells of hp-2dg mutant plants accumulate 14-fold higher amount of chlorophyll in mature-green stage and only 2.3-fold greater amount of total carotenoids (mostly lycopene) in ripe-red stage in comparison to their normal controls (Table VII). To explain this discrepancy, we analyzed chromoplast number and size in ripe-red pericarp tissues taken from hp-2dg mutant fruits and compared them to their isogenic counterparts. We found that the average chromoplast size of the mutant was 1.48 larger than its isogenic line (19.2 ± 0.4 and 13.0 ± 0.5 μm², respectively). Also, the chromoplast number in the mutant fruit pericarp was found to be 1.88-fold higher than the number of chromoplasts in its isogenic line (740.6 ± 64.9 and 393.6 ± 48.3 per cell, respectively). Thus, these results show a total of approximately 2.8-fold increase in chromoplast compartment size in the mutant compared to its normal counterpart. This fold increase corresponds to the 2.3-fold increase in total carotenoids between these genotypes at the ripe-red stage (Table VII).

Additionally, the above results indicate a 56.7% decrease in plastid number per cell in hp-2dg compared to a 7.1% decrease in its normal counterparts during chloroplast-chromoplast transition. These results suggest that the mutant fruits are subjected to a much stronger mechanism of plastid degradation upon ripening. This matter should be a subject of yet another comprehensive study because it has been long felt that the potential to produce carotenoids in hp-2dg mutant fruits should be higher.

We have previously suggested that increases in metabolites sharing antioxidative properties observed in hp tomato mutants represent responses of these light-hyperresponsive plants to light stress (Bino et al., 2005). Transcriptional up-regulation of stress-related genes displayed in hp-2dg mutant fruits in this study reinforce this statement (Table VI). The induction of transcripts involved in ROS scavenging, heat shock proteins (CHAPERONIN21), photosystem proteins repair machinery (FtsH METALLOPROTEASE), and...
EARLY LIGHT-INDUCED PROTEIN (ELIP) together with massive accumulation of secondary metabolites that share antioxidant properties also outline the reaction of normal plants exposed to light stress (Niyogi, 1999; Rossel et al., 2002). Interestingly, transcriptional induction of CHAPERONIN21 was shown to promote lycopene accumulation in the developing tomato fruits, probably by promoting the activity of carotenogenic enzymes (Neta-Sharir et al., 2005). On the other hand, transcriptional up-regulation of genes related to photosynthesis and plastid biogenesis is not usually observed in such light-stressed plants (Phee et al., 2004), indicating an additional or a separate role of DET1 on plastid proliferation and development in tomato fruits, at least those grown under normal light intensities. The increased number of chloroplasts in fruits of hp-2dg and hp-2j mutant plants is detectable as early as 8 DPA, indicating an early effect of a mutated DET1 on initiation of chloroplast proliferation during fruit development (Table VIII). Yet, the mechanism by which mutated DET1 increases plastid number and size is still to be identified. Recent studies point toward very complex mechanisms for chloroplast division and show that it is controlled by plastid- and nuclear-derived structural proteins (for review, see Aldridge et al., 2005) along with transcription factors (Fitter et al., 2002; Yasumura et al., 2005). Based on our results, we suggest that the molecular trigger mediating this process in DET1-mutated fruit cells should be studied at early stages of fruit development.

The results presented herein point toward a link between DET1, increased plastid biogenesis observed at early stages of fruit development, and the increased levels of carotenoids, vitamin C, vitamin E, and chlorophylls usually observed in mature fruits of hp-2dg and hp-2j mutants. Notably, RNA interference of tomato DET1 yielded both darker immature fruits and a significant increase in their carotenoid content upon ripening, using constructs driven by promoters specifically active during early stages of fruit development. On the other hand, use of E8 promoter, expressed from the early breaker stage of fruit development, did not affect fruit carotenoid levels (Davuluri et al., 2005). These latter results support our data positioning early plastid biogenesis machinery as a prerequisite for plastid-accumulating functional metabolites in ripe fruits.

Based on the results presented herein it is therefore reasonable to suggest that the identification of molecular mechanisms that drive the proliferation of chloroplasts in hp-2 mutants should be a target of future research in this field. We strongly believe that the identification and manipulation of genes that mediate the increase in plastid number at early fruit developmental stages could be a promising approach toward improving levels of functional metabolites in mature fruits.

CONCLUSION

The transcriptional profiling presented in this study points to genes related to plastid biogenesis and photosynthesis as the main group of genes that distinguish between hp-2dg fruit and their normal counterpart. These results are in agreement with the strong association obtained between plastid compartment size, calculated in fruit pericarp cells, and total carotenoids accumulating in this same tissue upon ripening. On the other hand, transcription of structural carotenogenesis genes was in effect unassociated with the high levels of carotenoids characterizing hp-2dg mutant pericarp fruit tissues. Because this genotype is characterized mainly by overproduction of plastid-accumulating metabolites, it is reasonable to conclude that the molecular machinery that drives plastid biogenesis is the main contributor to the significant increase observed in these metabolites in mature ripe fruits of hp-2dg genotypes. Our results further show that hp-2dg as well as hp-2j plants, both mutated at the DET1 gene, display a significantly increased plastid number and size starting at very early stages of fruit development (approximately 8 DPA). Together with a former study showing that transgenic modulation of DET1 is only effective when imposed at early fruit development stages (Davuluri et al., 2005), it can be further concluded that the initial molecular cue that increases plastid-accumulating metabolites in DET1 mutants should be traced at early stages of fruit development and is a requirement for the accumulation of these metabolites at later stages of fruit ripening.

MATERIALS AND METHODS

Plant Material and Experimental Design

A tomato (Solanum lycopersicum) line (n-935) homozygous for the hp-2j mutation and its nearly isogenic normal counterpart were used for the microarray analysis. Line n-935 was developed at the Volcani Center as a determinate high-lycopene processing line (Levin et al., 2003). Although fully isogenic indeterminate lines for the hp-2j mutation were available to us (LA2451 and LA3007 from the Tomato Genetics Cooperative, University of California, Davis), we preferred the n-935 line due to its self-pruning nature, a plant habit characterized by concentrated flowering, as well as fruit anthesis and ripening. Also, tomato plants sharing such plant habit do not require special handling such as cutting and stripping during growth, thus ensuring better reproducibility between experiments.

Representative plants of line n-935 and their normal counterparts were planted in a randomized block design (three blocks, five plants in each block) in an environmentally controlled greenhouse at the Volcani Center, Bet Dagan, Israel, during the winter seasons of 2004 and 2005 (minimal temperature kept at 15°C with no supplemental light). At each of three developmental stages (mature-green, breaker, and early red), a single fruit was harvested from a randomly chosen plant of each genotype within each block. In each harvest different plants in each block were taken to represent each genotype. Following harvest, equal pericarp samples were cut from the equator region of each fruit. These samples were pooled according to genotype prior to RNA extraction, allowing equal representation of each block in the final sample. A total of three harvests taken from each genotype were eventually analyzed, each harvest representing a biological repeat.

Confocal microscopy studies were carried out on developing and mature-green fruits harvested from hp-2dg and hp-2j mutant plants, as well as their normal counterparts. These plants were grown in a randomized block design (two blocks, six plants in each block) in a greenhouse during the winter season of 2005 (minimal temperature kept at 15°C with no supplemental light) and in a screen house during the summer season of 2006 (with no additional heating and supplemental light). From each of the two blocks, single representative fruits were harvested from three to five different plants of each genotype. Chlorophyll and carotenoid contents were analyzed in a sample of 20 fruits.
RNA Extraction and Amplification

Total RNA was isolated from approximately 1 g of pericarp tissue of mature-green, breaker, and early red fruits harvested from hp-2h/hp-2h plants and their normal counterparts using Qiagen RNeasy Midi kit (Qiagen). RNA isolation was according to the manufacturer’s recommendations with the following modifications: (1) tissue was pulverized in RLT buffer containing β-mercaptoethanol and (2) RNA samples were treated on column for 1 h, with DNase I using Qiagen RNase-free DNase set. Each RNA sample was subjected to mRNA amplification procedure using MessageAmp aRNA kit (Ambion). Three micrograms of total RNA were used in each amplification reaction.

Microarray Experiment Analysis

Transcriptional profiling of tomato pericarp tissue of hp-2h/hp-2h plants was obtained using direct comparisons with their normal counterparts in each developmental stage separately. Two-color hybridizations were carried out to the TOM1 microarray slide (http://bti.cornell.edu/CGEP/CGEP.html). Data from this experiment have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) and are accessible through Gene Expression Omnibus Series with accession number GSE6041.

The TOM1 chip, previously used in other studies (van der Hoeven et al., 2002; Albo et al., 2004, 2005; Fei et al., 2004; Sagi et al., 2004; Bar-Or et al., 2005, 2006), contains 12,501 cDNA spots. However, following resequencing of those cDNA clones, 4,497 clones overlapped and could be assigned to the same unigene (Cornell site: http://bti.cornell.edu/CGEP/CGEP.html). Our analyses are therefore initially based on these 4,497 confirmed unigenes. Three micrograms of amplified mRNA products were subjected to reverse transcription and then labeled with Cy3 and Cy5 (Amersham Biosciences) by the indirect amino-allyl method (Invitrogen). Hybridization of labeled targets to the microarray slide TOM1 was carried out at 42°C for 14 to 16 h using a covered hybridization oven (Amersham Pharmacia). Microarray slides were washed seven times for 10 min each in 2× SSC/0.1% SDS at room temperature, in 0.2× SSC/0.1% SDS at room temperature twice, in 0.2× SSC at room temperature twice, and once in 0.2× SSC at 42°C. Excess fluids were drained from the arrays by centrifugation (5 min at 450g).

For each developmental stage (mature green, breaker, and early red), four hybridizations were carried out, representing three independent biological repeats and one technical repeat of swapped dyes. Separate images for each fluorescence dye were acquired using ScanArray 4000 genetic analyzer (GSI Lumonics [Packard/Perkin Elmer]). The analyzer scan was carried out at 10 μm resolution per pixel, adjusting the photomultiplier and laser power to achieve an optimal distribution of signals with minimal saturation. Quantification was carried out using QuantArray version 3 software, applying histogram method (Packard BioScience). Data analysis was performed using Genespring 7.2 software (Silicon Genetics) applying per-spot and per-chip histograms (Packard BioScience). Data analysis was performed using Genespring 7.2 software (Silicon Genetics) applying per-spot and per-chip histograms method (Packard BioScience). Data analysis was performed using Genespring 7.2 software (Silicon Genetics) applying per-spot and per-chip normalization methods for each hybridization. Lowess curve was fit to the log-intensity versus log-ratio plot. Forty percent of the data was used to calculate the Lowess fit at each point. This curve was used to adjust the value of the normal genotype for each measurement (control channel). If the control channel was lower than 10 then 10 was used instead. In all data tables obtained from each hybridization, low-quality data were identified and discarded when signal-to-noise ratio was <2. Statistically reliable data were obtained applying filter on confidence (P(0) = 0.05, Benjamini-Hochberg false discovery rate = 0.05). Significantly altered genes in every developmental stage were then filtered out from the statistically reliable data based on fold change (>2 for over- and <0.5 for underexpressed). Only array spots containing clones with known 5′ and 3′ sequence that could also be assigned to a certain unigene at value < 5C were further used for ontology assignment. Ontology terms were assigned to genes using the Solanaceae Genomics Network (SGN; http://www.sgn.cornell.edu) and The Institute of Genomic Research (http://www.tigr.org/) databases. SGN-U sequences derived from SGN database were used as queries in BLAST analysis against the tomato database deposited at The Institute of Genomic Research and the corresponding Gene Ontology terms, based on biological process or molecular function, were retrieved.

The MAPMAN software was further used to obtain a visual display of our final microarray data onto diagrams representing metabolic pathways (Thimm et al., 2004). Originally developed for Arabidopsis (Arabidopsis thaliana), MAPMAN was successfully implemented in analysis of TOM1 array-generated data from several Solanaceous species (Urbanczyk-Wochniak et al., 2006). Prior to the analysis by MAPMAN, data were transformed to their natural logarithm.

Real-Time Quantitative PCR Reactions

The same total RNA samples extracted for the microarray experiment were used to synthesize cDNA templates required for the qRT-PCR analysis. cDNA synthesis was carried out using iScript cDNA synthesis kit (Bio-Rad). Gene-specific primers were designed using the Primer Express software (Applied Biosystems). qRT-PCRs were performed using SYBR GREEN PCR master mix (Applied Biosystems) on ABI Prism 7000 cycler (Applied Biosciences). The cycling conditions were 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. The genes with their SGN unigene code and the forward (F) and reverse (R) primers designed for each gene are presented in Supplemental Table S1. Expression levels of genes of interest (target genes) in hp-2h/hp-2h and control samples were determined relative to that of tomato L185 RNA (Kolits and Bird, 2000) and two technical replicates were performed in every biological repeat.

Microscopy and Image Analyses

Prior to the microscopy observations, fruits were sliced with sharp scalpels on the equatorial line providing two to three slices of 0.3 to 0.5 mm thickness per fruit. Each slice contained all the tissue layers, such as epidermis, endocarp, outer, and inner mesocarp, according to Waters et al. (2004). The slices were placed on microscope glass in a drop of sterile water and covered with cover glass.

Chloroplast observations and image acquisitions were performed using the OLYMPUS IX 81 (Japan) inverted laser scanning confocal microscope (FLUOVIEW 500) equipped with a 488 nm argon-ion laser. Chlorophyll was excited by 488 nm light and the emission was collected through BA 660 filter. The images were color coded red for chlorophyll autofluorescence. Confocal optical sections were obtained at 1 μm increments. Chloroplasts were then counted in five to 15 individual cells, randomly chosen in each slice from the outer and inner mesocarp sectors, avoiding the regions proximal to vasculature. The magnification can be increased by zooming the scanning laser beam on a smaller area of the object. The transmitted light images were obtained using Nomarski differential interference contrast. Image analyses were carried out using ImageJ 1.37 v software (National Institutes of Health) according to manufacturer’s protocols and examples. Plan area measurements were used to assess cell and chloroplast sizes.

Chromoplast observations were carried out on a Leica DM LB microscope (Leica) and light images were acquired using a Leica DC-200 digital camera.

Carotenoid Extraction, Identification, and Quantification

All chemicals and solvents were purchased from Sigma. Carotenoid content was analyzed in isolated pericarp tissue removed from 20 ripe-red fruits of hp-2h/hp-2h and hp-2/hp-2 plants and their normal isogenic counterparts. All steps were performed in darkness or under gold fluorescent light to avoid possible photodegradation of carotenoids. All samples were processed in an electric blender to a fine puree. Two sample aliquots of about 0.5 g were subsequently taken from each sample, representing two technical repeats of each genotype in each block. Total carotenoid extraction included rigorous shaking in 8 mL of hexane:acetoneethanol (50:25:25) for 5 min, addition of 1 mL KOH in water (25% w/v), vortexing and shaking for 5 min, and then addition of 1 mL of NaCl in water (25% w/v), vortexing and shaking for 5 min. Subsequently, 8 mL of water were added, the samples were mixed by vortex and incubated for 10 min on the bench in dark. Finally, the upper phase was collected, reextracted with additional 2 mL of hexane, and the combined extract was analyzed for absorbance in the wavelength range 300 to 600 nm by Cary50Bio spectrophotometer (Varian) against the appropriate blank (hexane). Lycopene content was calculated using the formula: lycopene content (μg/g fresh weight) = Abs. (A274) × 10,000 × 0.5 (or dilution factor) × [3,450 (specific absorption coefficient of lycopene in cm-1 × exact weight in grams)], based on Davis (1965).

For HPLC analysis (Tadmor et al., 2000), the combined hexane solution was evaporated to dryness in a Savant SpeedVac apparatus and resuspended in
Chlorophyll Extraction and Quantification

Chlorophyll was extracted from pericarp sections (approximately 1.3 g) of fruits of hp-2dg/hp-2j, hp-2j/hp-2, and their normal counterparts via incubation in 10 mL of dimethyl formamide (Fluka) for 72 h at 4°C in complete darkness. Absorbance of extracts was measured using a UV-2401PC spectrophotometer (Shimadzu). Total chlorophyll content was estimated using the method of Inskrip and Bloom (1985).

Statistical Analyses of Additional Quantitative Data

Chloroplast characteristics as well as fruit chlorophyll and lycopene contents were analyzed using a two-way ANOVA, focusing on the genotype effect. All these analyses were carried out with the JMP statistical discovery software (SAS Institute).

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