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ARTICLE

Characterization of the *INTENSE PIGMENT* Tomato Genotype Emphasizing Targeted Fruit Metabolites and Chloroplast Biogenesis

Noga Lavi,[†] Yaakov Tadmor,[†] Ayala Meir,[†] Avital Bechar,[§] Michal Oren-Shamir,[†] Rinat Ovadia,[†] Moshe Reuveni,[†] Sahadia Nahon,[†] Haviva Shlomo,[†] Lea Chen,[†] and Ilan Levin*,[†]

[†]Institute of Plant Sciences and [§]Institute of Agricultural Engineering, Agricultural Research Organization, Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

The tomato *INTENSE PIGMENT* (*IP*) genotype is characterized by intense visual pigmentation of unripe and ripe fruits, not thoroughly analyzed thus far. This study was therefore designed to analyze key morphologic, metabolomic, and photomorphogenic phenotypes of this genotype in comparison to its near-isogenic normal counterpart and to evaluate its significance relative to other tomato mutants known for increased fruit pigmentation. The *IP* genotype produced smaller and darker red fruits, and a substantially increased chloroplast biogenesis was found in its green fruit and leaf tissues. Ripe-red fruits of the *IP* genotype produced 34−38% more soluble solids and up to 62.6% more carotenoids, but no differences were found in the concentration of flavonoid compounds in its peel tissue. The *IP* genotype was found to display a greater hypocotyl inhibition response to blue and yellow light, but a more prominent and novel response to total darkness. As a whole, the *IP* genotype exhibited highly desirable traits, making it a valuable genotype for tomato breeders attempting to introduce functional and taste qualities into tomato fruits.

KEYWORDS: Tomato; *INTENSE PIGMENT*; carotenoids; plastid biogenesis; photomorphogenesis

INTRODUCTION

Fruits and vegetables are a source of nutritional compounds indispensable for maintaining a healthy life style. These positive nutritional effects may arise from secondary metabolites such as flavonoids and carotenoids (*1*, *2*).

The tomato fruit is a good source of carotenoids, such as lycopene and β-carotene (*3*), and a potential source of flavonoid compounds as well (*4*). Studies have suggested that the consumption of tomatoes and tomato-based products reduces the risk of chronic diseases such as cancer and cardiovascular diseases. These protective effects have been mainly associated with carotenoids, the major phytochemicals present in the tomato fruit (*5*). The most abundant carotenoid in ripe-red tomato is lycopene, followed usually by much lesser amounts β-carotene, phytoene, phytofluene, ξ-carotene, γ-carotene, neurosporene, and lutein (*1*, *2*). Lycopene was found to be highly protective against potential damage caused by singlet oxygen and to prevent cardiovascular diseases and breast, prostate, and colon cancers (*5*, *6*). In addition, lycopene as well as other carotenoids has many industrial applications propelling a continuously growing market with an estimated yield of 100 million tons and a value of about U.S. $935 million per annum (*1*, *2*).

*Author to whom correspondence should be addressed (telephone: +972 3 9683477; fax: +972 3 9669642; e-mail vclevini@volcani.agri.gov.il).

Efforts have been invested in increasing the content and diversifying the phytonutrients, such as carotenoids and flavonoids, in the tomato fruit (*1*, *2*, *4*, *7*). These efforts rely on transgenic and nontransgenic approaches, and principal among the latter approaches is the utilization of photomorphogenic mutants (*2*, *4*).

"Photomorphogenesis" refers to the array of interactions by which plants modulate their growth and development in response to light and is affected by several sets of genes. Mutations in any of these light signaling genes may cause a significant change in the developmental program of plants, which is often accompanied by modulated gene expression and profound effects on cell differentiation and plastid biogenesis (*2*, *8*, *9*).

The light-responsive *high pigment* (*hp*) mutations *hp-1*, *hp-1*^{w}, *hp-2*, *hp-2*^{j}, and *hp-2*^{dg} are the most noted and studied photomorphogenic mutations thus far described in tomato. Mutant plants carrying these mutations display shorter hypocotyls, particularly under suboptimal light conditions, and, more importantly, significantly higher levels of lycopene in addition to an array of fruit phytonutrients than their isogenic or near-isogenic normal counterparts (*10*−*12*). Consequently, introgression of these mutations into elite processing and fresh-market cultivars has become an important strategy in breeding projects directed to increase fruit functional quality (*2*, *11*).

Recent studies have shown that *hp* mutations map to *DEETIOLATED1* (*DET1*) and the gene encoding UV-DAMAGED DNA BINDING PROTEIN 1 (*DDB1*), two evolutionary

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conserved regulatory genes active in light signaling, known also as photomorphogenesis (*10*, *11*, *13*, *14*). It has been known for 50 years that incident light can directly regulate the tomato fruit pigmentation process (*15*), but to the best of our knowledge such studies have not been significantly elaborated since then. The recently identified genetic link between the light signaling *DET1* and *DDB1* genes and increased pigmentation in tomato fruits strongly suggests that manipulation of light signal transduction machinery may be particularly effective in modulating an array of fruit phytonutrients (*2*, *11*, *16*).

Studies carried out on *hp-1* and *hp-2^{dg}* mutant plants have suggested that plastid biogenesis is the major determinant which drives the increase in fruit phytonutrients in these mutants (*9*, *17*, *18*). Interestingly, this concept was also lately documented in the characterization of tomato plants mutated at the *ZEAXANTHIN EPOXIDASE* (*ZE*) gene (*19*).

The *INTENSE PIGMENT* (*IP*) genotype originated from a cross between the wild species *Solanum chmielewskii* and the cultivated tomato (*Solanum lycopersicum*). It has been characterized as having both increased content of soluble solids (*20*−*22*) and increased visual pigmentation of its immature as well as mature fruits (*23*). Three segments originating from the wild species have been mapped to chromosomes 7 and 10 in the *IP* genotype, and their effects on increased soluble solids in mature fruits were thoroughly analyzed (*21*, *22*, *24*, *25*). Unlike the recessive *hp* mutations, which encode repressors of phytochrome signal amplification, the *IP* genotype was characterized as dominant (*20*) and was implicated in a promotion of phytochrome signal amplification (*26*). Similarly to *hp* mutants, the *IP* genotype was also found to display a hypocotyl inhibition response under red and far-red light, but with a preferentially enhanced response in hypocotyl anthocyanin accumulation under blue light (*23*, *26*). These responses render *IP* photomorphogenic, similarly to the five aforementioned *hp-1* and *hp-2* mutations. However, to the best of our knowledge, very little else is known about the *IP* genotype. The purpose of this study was to fill this knowledge gap and to extend the analysis of the *IP* genotype. The fact that *IP* incorporates increases in pigmentation and sugars, two major fruit quality traits, merits such analysis.

This study provides, for the first time, a comprehensive analysis of the *IP* genotype, displaying its key morphologic, metabolomic, and photomorphogenic phenotypes. This comprehensive analysis enables an improved evaluation of this genotype, on its own and in relation to the tomato *hp* mutants, and provides a solid framework for further genetic studies.

MATERIALS AND METHODS

Plant Material and Experimental Layout. This study presents representative data obtained from series of experiments that took place at the Volcani Center, Bet Dagan, Israel, during the summer (seedlings transplanted first week of May) and winter (seedlings transplanted first week of October) seasons of 2003 through 2007. In each experiment, tomato (*S. lycopersicum*) *IP* line LA1563 and its normal near-isogenic line LA0816 (the open-pollinated cv. 'F-145-22-8') were grown in a randomized block design (three blocks, 5−10 plants in each block). During summer, plants were grown under an insect-proof 50 mesh net covered with a black screen allowing 70% light transmittance. During winter, plants were grown in an environmentally controlled glass greenhouse where the minimal temperature was kept at 18 °C with no supplemental light. Several attempts to grow the genetic material in the open field failed due to extreme fruit bleaching observed in both genotypes under the relatively high temperatures and light intensities characterizing Israeli summers. Seeds of LA1563 and LA0816 were obtained from Tomato Genetics Resource Center (TGRC at http://tgrc.ucdavis.edu/). The *IP* line LA1563 was developed from repeated backcrossing of the wild species *S. chmielewskii* to *S. lycopersicum* cv. 'VF36' (BC_2) and later to cv. 'VF-145-22-8' (BC_5), the latter being the normal isogenic counterpart of LA1563 in this study (*20*, *21*).

Fruit Weight and Total Soluble Solids (TSS). Twenty representative fully red-ripe fruits, 15 days postbreaker, were harvested from each genotype in each block, weighed, and minced to a fine puree in a blender. TSS measurements, based on °Brix, were carried out on fruit juice obtained from the combined puree using a palette refractometer (Atago Co., Tokyo, Japan).

Morphological Analysis of Fruit and Leaf Cells. External leaf observations were carried out in summer 2006 using a Leica DM LB stereomicroscope, and light images were acquired using a Leica DC-200 digital camera (Leica Microsystems, Wetzlar, Germany).

Chloroplast observations and image acquisitions of fruit and leaf tissues were carried out in summer 2007 using the Olympus 1X-81 (Olympus Co., Tokyo, Japan) inverted laser scanning confocal microscope (Fluoview 500) equipped with a 488 nm argon-ion laser and a UPlanApo10X NA 0.4 objective. The 488 nm line of an argon laser was used for chlorophyll excitation, followed by a BA660IF emission filter. The images were color coded red for chlorophyll autofluorescence. Transmitted light images were acquired by means of Nomarski differential interference contrast microscopy. Confocal optical sections were obtained at 1 μm increments.

Confocal microscopy studies were carried out on mature-green fruits and on leaves harvested from *IP* plants, as well as their normal near-isogenic counterparts: (1) For fruit chloroplasts, fruits were sliced with a sharp scalpel on the equatorial region, providing two to three slices of 0.3−0.5 mm thickness per fruit. Each slice contained all of the pericarp tissue layers, including epidermis, outer and inner mesocarp (according to ref *27*), and gel tissue. The fruit slices were placed on a microscope slide in a drop of sterile water and covered with a coverslip; 10 photographs were taken from each tissue (outer mesocarp, inner mesocarp, and gel tissue). (2) For leaf chloroplasts, both mid and top leaves of three *IP* plants and their near-isogenic counterparts were sampled, one leaf per region. A 1 cm^2 leaf section was placed on a microscope slide in a drop of sterile water and covered with a coverslip, and five photographs were taken from each leaf section.

Detailed images of fruit and leaf tissues and their chloroplasts were obtained with an Olympus IX-81 fully automated laser scanning confocal microscope (see above). The image resolution was 912 × 912 pixels with a color depth of 24 bit. For a fruit tissue, each pixel represented 1.73 μm^2 and the entire image covered 1.65 mm^2, and for a leaf tissue, each pixel represented 0.048 μm^2 and the entire image covered 0.046 mm^2. The images were saved in Tagged Image File format (TIF). An image analysis algorithm was developed with the image-processing toolbox of Matlab 7 (TheMathWorks, Natick, MA). The 24-bit original images were divided into three bands of 8-bit images: red, green, and blue. A threshold value was determined to distinguish between cellular tissues with chlorophyll emission from others by creating a binary image in which white areas represent the presence and location of chlorophyll and black areas represent other cellular parts of fruits and leaves that do not contain chlorophyll. Two performance measures were obtained from the processed images: (1) area ratio, that is, the ratio between the chlorophyll-containing area and the entire area covered by the image; (2) average intensity, that is, the average intensity value of the chlorophyll-containing area on a 0−1 scale. Thus, the average area ratios presented in this paper represent average plastid compartment size, whereas the average intensity levels represent chlorophyll concentration in chloroplasts.

Chlorophyll Content of Fruits and Leaves. Chlorophyll was extracted from disks of fruit pericarps (10 mm in diameter, approximately 1.3 g) and leaves (10 mm in diameter, approximately 0.25 g) taken from *IP* plants and their normal near-isogenic counterparts via incubation in 10 mL of *N*,*N*-dimethylformamide (Fluka, Gillingham, U.K.) for 72 h at 4 °C in complete darkness (*9*). Absorbance of extracts was measured using a UV-2401PC spectrophotometer (Shimadzu Co., Kyoto, Japan). Total chlorophyll content was calculated using the method of Inskeep and Bloom (*28*).

Carotenoid Profiling of Fruits and Leaves. Carotenoid content was analyzed in 20 representative ripe-red fruits or 5 leaves taken from *IP* plants and their near-isogenic counterparts from each of three blocks. All steps of sample preparation were carried out in darkness or under gold fluorescent light to avoid possible photodegradation of carotenoids.

Fruit samples were processed in an electric blender to a fine puree, whereas leaf samples were ground in liquid nitrogen. Extraction, determination, and quantification of carotenoid compounds were described in detail (*9*). In essence, extraction included vigorous shaking of an accurately weighted sample in 8 mL of hexane/acetone/ethanol (50:25:25, v/v/v) for 5 min, followed by saponification through an additional shaking for 5 min after the addition of 1 mL of KOH in H_2O (80%, w/v). Phase separation was achieved after the addition of 1 mL of NaCl in H_2O (25%, w/v) and vortexing, which was then followed by the addition of 8 mL of cold H_2O. The mixture was again vortexed for 5 min followed by 10 min of incubation in darkness for phase separation. Finally, the upper phase was collected, while the lower phase was re-extracted with an additional 2 mL of hexane, and the combined extracts were completely dried with a Savant SC110A Speed Vac Plus (Thermo Scientific, Cambridge, MA). The dried samples were resuspended in 400 μL of acetonitrile/methanol/dichloromethane (45:5:50, v/v/v). Forty microliters of filtered samples was fractionated on a C18 Nova-Pak (Waters, Milford, MA) column (250 × 4.6 mm i.d.; 60 Å; 4 μm) and a Nova-Pak Sentry Guard cartridge (Waters) with a 2996 Waters HPLC equipped with a Waters PDA detector 996 using a mobile phase containing 10% of 0.5% triethylamine in acetonitrile, 3% methanol, and a gradient starting with 87% acetonitrile and changing to 77% while increasing dichloromethane from 0 to 10%. These conditions were kept for an additional 15 min. The flow rate of the mobile phase was 1.5 mL/min, whereas the column temperature was 30 °C. The detector measured from 265 to 700 nm at a sampling rate of 1 spectrum/s and an optical resolution of 2.4 nm. Carotenoids were identified by comparison of retention times, co-injection spiking, and comparison of their UV−visible spectra with authentic standards (Sigma, St. Louis, MO, U.S.). Quantification was performed by integrating the peak areas of the HPLC results using Millennium chromatography software (Waters) assisted by the previously prepared standard curves.

Flavonoid Profiling of Fruits. Samples of fresh tomato skins (0.1−0.3 g) were taken from the equator region of ripe-red fruits and extracted with acidic methanol as previously described (*4*). Flavonoid composition was determined using a high-performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) equipped with an LC-10AT pump, an SCL-10A controller, and an SPD-M10AVP photodiode array detector. Extracts were loaded onto an RP-18 column (Vydac 201TP54) and separated at 27 °C with the following solutions: (A) H_2O, pH 2.3, and (B) H_2O/acetonitrile/acetic acid (107:50:40, v/v/v), pH 2.3. Solutions were applied as a linear gradient from a ratio of 4:1 (A:B) to 3:7 over 45 min and held at a ratio of 3:7 for an additional 10 min at a flow rate of 0.5 mL/min. Flavonoids were identified by comparing both the retention time and the absorption spectrum at 250−650 nm with those of standard purified flavonoids (Apin Chemicals, Abingdon, U.K.; Sigma).

Photomorphogeic Response to Various Light Conditions. Seeds of both *IP* genotype and its near-isogenic counterpart were sown in small pots on a regular planting soil and covered with vermiculite. Three pots containing seeds of each genotypes were then exposed to white, yellow, or blue light (12 h light/12 h dark) in an environmentally controlled growth chamber (25 °C). Three pots containing seeds of each genotype were also placed in total darkness under the same conditions. Eleven-day-old seedlings were removed from the pots, and their hypocotyl lengths were individually measured.

Statistical Analyses. Statistical analyses were carried out with the JMP statistical discovery software (SAS Institute Inc., Cary, NC). The model for most analyses included effects related to block and genotype and in some analyses a seasonal effect as well. In most cases no statistically significant block effect was found.

RESULTS

Fruit Weight, Total Soluble Solids, and Lycopene Content. Average weight, TSS, and lycopene concentration of ripe-red fruits harvested from the *IP* genotype and its normal near-isogenic counterpart during winter (2003/2004) and summer (2004) seasons are presented in **Table 1**. Season significantly affected average fruit weight, with winter fruits being significantly smaller than summer fruits. In winter, the genotype significantly affected both fresh weight and TSS, with *IP* fruits being significantly smaller and having significantly higher TSS than their near-isogenic counterparts. In summer, TSS was similarly affected by the genotype, but *IP* fruits and their near-isogenic counterparts were statistically similar in size. Fruit lycopene concentration significantly differed between genotypes in both seasons, but a much larger relative difference between genotypes, presented as change (%) in **Table 1**, was observed in the summer. Interestingly, despite the statistically significant differences observed in average fruit weight of the two genotypes between seasons, TSS levels remained constant in the two genotypes regardless of season. Lycopene levels, on the other hand, displayed a negative relationship with average fruit weight, with smaller fruits having higher lycopene levels and vice versa. Still, the increase in average fruit lycopene concentration attributed to the *IP* genotype was much larger than the reduction observed in its average fruit weight in both seasons, indicating that the lycopene increase in *IP* fruits was not the result of reduction in fruit weight.

Table 1. Effects of Season and Genotype on Fresh Weight, Total Soluble Solids (TSS), and Lycopene Content of Ripe-Red *IP* Fruits in Comparison to Their Near-Isogenic Counterparts[a]

season	genotype	fresh wt (g)	TSS (°Brix)	lycopene (μg g^{-1} of FW)
winter	*IP/IP*	71C ± 4	5.8A ± 0.1	112A ± 4
	+/+	84B ± 4	4.3B ± 0.2	76C ± 4
change (%)		−15	+35	+47
summer	*IP/IP*	109A ± 3	5.8A ± 0.1	94B ± 4
	+/+	122A ± 3	4.2B ± 0.2	55D ± 3
change (%)		−11	+38	+71

[a] Values represent mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between genotypes and seasons for each trait separately.

Table 2. Carotenoid Profile of Ripe-Red *IP* Fruits in Comparison to Their Near-Isogenic Counterparts[a]

	genotype		
carotenoid	*IP/IP*	+/+	change (%)
lycopene	75.20A ± 3.59	46.09B ± 2.04	+63.2
β-carotene	3.40A ± 0.05	2.68B ± 0.19	+26.9
phytoene	2.14A ± 0.10	0.97B ± 0.06	+120.6
phytofluene	0.92A ± 0.07	0.47B ± 0.05	+95.7
lutein	0.48A ± 0.06	0.42A ± 0.06	+14.3
ζ-carotene	0.22A ± 0.02	0.15B ± 0.01	+46.7
total	82.37A ± 3.45	50.67B ± 2.25	+62.6

[a] Values represent average carotenoid concentrations (in μg g^{-1} of FW) ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between genotypes for each carotenoid compound separately.

Carotenoid Profiling of Fruits and Leaves. Due to higher effect of the *IP* genotype on fruit lycopene levels during the summer season, fruits and leaves harvested from this season were subjected to targeted HPLC carotenoid profiling. **Tables 2** and **3** display the results obtained from fruits and leaves, respectively. Total content of carotenoids in the ripe-red fruits of the *IP* genotype were about 62.6% higher than in their near-isogenic counterparts. Except for lutein, all fruit carotenoids were significantly increased in the *IP* genotype compared to its near-isogenic counterpart (**Table 2**).

Total carotenoid concentration in leaves of the *IP* genotype was 22.8% higher than in leaves of its near-isogenic counterpart.

Table 3. Levels of Major Carotenoid Compounds Detected in Leaves Taken from the *IP* Genotype in Comparison to Their Near-Isogenic Counterparts[a]

	genotype		
carotenoid	*IP/IP*	+/+	change (%)
lutein	91.3A ± 11.6	73.0A ± 3.5	+25.1
β-carotene	66.7A ± 7.7	54.3A ± 2.2	+22.8
γ-carotene	3.3A ± 0.3	2.3A ± 0.3	+43.5
phytoene	2.7A ± 0.3	3.3A ± 0.3	−18.2
total	163.3A ± 19.8	133.0A ± 5.1	+22.8

[a] Values represent average carotenoid concentrations (in μg g^{-1} of FW) ± SE. No significant differences were observed in levels of carotenoid compounds between genotypes.

However, this difference was not statistically significant. With the exception of phytoene, which was insignificantly reduced, all other major carotenoid compounds, measured in leaves, displayed higher but statistically insignificant levels in the *IP* genotype (**Table 3**).

Microscope Analysis of Fruits and Leaf Tissues. An example of confocal microscopy image analysis obtained from *IP* fruits and leaves is shown in **Figure 1** (**A1**−**4** and **B1**−**4**, respectively). Panels **A1** and **B1** of **Figure 1** display transmitted light images of fruits and leaves, respectively, with chlorophyll emitting red light. Panels **A2** and **B2** of **Figure 1** illustrate the red image intensity level for fruits and leaves, respectively; bright colors represent high intensity and vice versa. The binary images in which the white areas represent the presence and location of chlorophyll and the black areas represent other cellular parts of fruits and leaves that do not contain chlorophyll are presented in **Figure 1**, panels **A3** and **B3** for fruits and leaves, respectively. A histogram depicting the intensity level of a saturation image with a threshold value separating chlorophyll from other parts of the image is presented in panels **A4** and **B4** of **Figure 1** for fruits and leaves, respectively.

Mature-green (**Figure 2**) and ripe-red fruits of the *IP* genotype were visibly darker than their near-isogenic counterparts. Confocal microscopy of outer and inner mesocarp as well as gel tissues of mature-green fruits, harvested in the summer season 2006, revealed that the relative area occupied by chloroplasts in these tissues was significantly higher in *IP* plants than in their near-isogenic counterparts (**Figure 2**). However, multiple slides taken from multiple fruits in two summer seasons (2006 and 2007) revealed that whereas the differences in chloroplast coverage between genotypes in the outer mesocarp and gel tissues were highly consistent, differences observed in inner mesocarp cells were not as consistent, displaying no statistical differences between genotypes (**Table 4**). Furthermore, chlorophyll intensity levels in fruit tissues differed only slightly between genotypes. Because the intensity measurements displayed in **Table 4** point to chlorophyll content in chloroplasts, it can be concluded that the two genotypes do not substantially differ in chlorophyll content per chloroplast in mature-green fruit tissues.

Leaves of the *IP* genotype were visibly greener and covered with larger and denser glandular trichomes than their normal isogenic counterparts (**Figure 3**). Confocal microscopy of leaves revealed that similarly to fruit tissues, the area chloroplasts occupy in the leaf tissue is substantially greater in the *IP* genotype than in its isogenic normal counterpart, whereas intensity levels were very similar, indicating that the content of chlorophyll in leaf chloroplasts is in effect the same in the two genotypes (**Table 4**). In addition, a striking difference in the size of leaf mesophyll cells between *IP* genotype and its isogenic counterpart is clearly evident (**Figure 3**). The mesophyll cells of the *IP* genotype were smaller than those of their normal isogenic counterparts.

Chlorophyll Content of Fruits and Leaves in Relation to Microscope Observations. Chlorophyll concentrations in both mature-green fruit pericarps and in green leaves of *IP* plants were significantly higher than in their near-isogenic counterparts by approximately 30% (**Table 5**). Detailed analysis of the fruit-tissue images obtained by the confocal microscope, displayed in **Table 4**, showed that these results are in general more correlative to average area occupied by chloroplasts rather than to average intensity levels obtained for both tissues. It also appears that in fruit pericarp tissues the average area occupied by chloroplasts in the outer mesocarp is the main contributor to the higher chlorophyll levels observed in the *IP* genotype.

Flavonoid Profiling of Fruits. There were no significant differences in levels of major flavonoid compounds in peel tissue of ripe-red tomato fruits of the *IP* genotype in comparison to their near-isogenic counterparts. The levels of selected flavonoid compounds in peel tissues of the two genotypes are presented in **Table 6**.

Photomorphogenic Response to Various Light Conditions. When grown under yellow or blue light as well as in total darkness, the seedlings of *IP* showed a statistically significant hypocotyl inhibition response, whereas under white light there was no such response. Interestingly, the strongest response was under total darkness (**Table 7**).

DISCUSSION

This study describes key morphologic, metabolomic, and photomorphogenic phenotypes of the tomato *IP* genotype in comparison to its near-isogenic counterparts and evaluates its significance relative to *hp* mutant genotypes known for their highly increased fruit pigmentation. The *IP* genotype differed significantly in most measured phenotypes from its near-isogenic counterparts−most of these phenotypes resemble *hp* mutant genotypes, but with a few important exceptions.

The *IP* genotype produced smaller (see also ref *25*) and darker red mature fruits than their near-isogenic counterparts. The mature-green fruits of the *IP* genotype and their leaves were also greener than those of their near-isogenic counterparts. Various *hp* mutants are known to exhibit similar yet more pronounced phenotypes; their mature-green fruits tend to be darker green and their ripe-red fruits are usually darker red than those of *IP* fruits (personal observations; see also refs *2*, *9*, and *29*). In addition and similarly to *IP*, *hp* mutant fruits usually yield significantly smaller fruits in comparison to their corresponding isogenic counterparts.

The *IP* genotype produced more soluble solids and more carotenoids, including lycopene, in ripe-red fruits and also more chlorophyll in both mature-green fruits and leaves. Increased soluble solids in the ripe-red fruits of the *IP* genotype, previously highly elaborated (*21*, *22*, *24*, *25*), was reconfirmed in this study both in summer and in winter seasons, demonstrating that this trait is a highly consistent quality trait of the *IP* genotype.

Ripe-red fruits harvested from the *IP* genotype were found to accumulate up to 62.6% more carotenoids than their near-isogenic counterparts. In both genotypes the primary carotenoid was lycopene, accounting for about 90% of the total carotenoids. Most other carotenoids were significantly increased, 27−120%, in ripe-red fruits of the *IP* genotype, suggesting that lycopene is just one in an array of carotenoids which accumulate in the *IP* genotype. Measured levels of total carotenoids per gram of fresh weight in the leaves of both genotypes were approximately twice

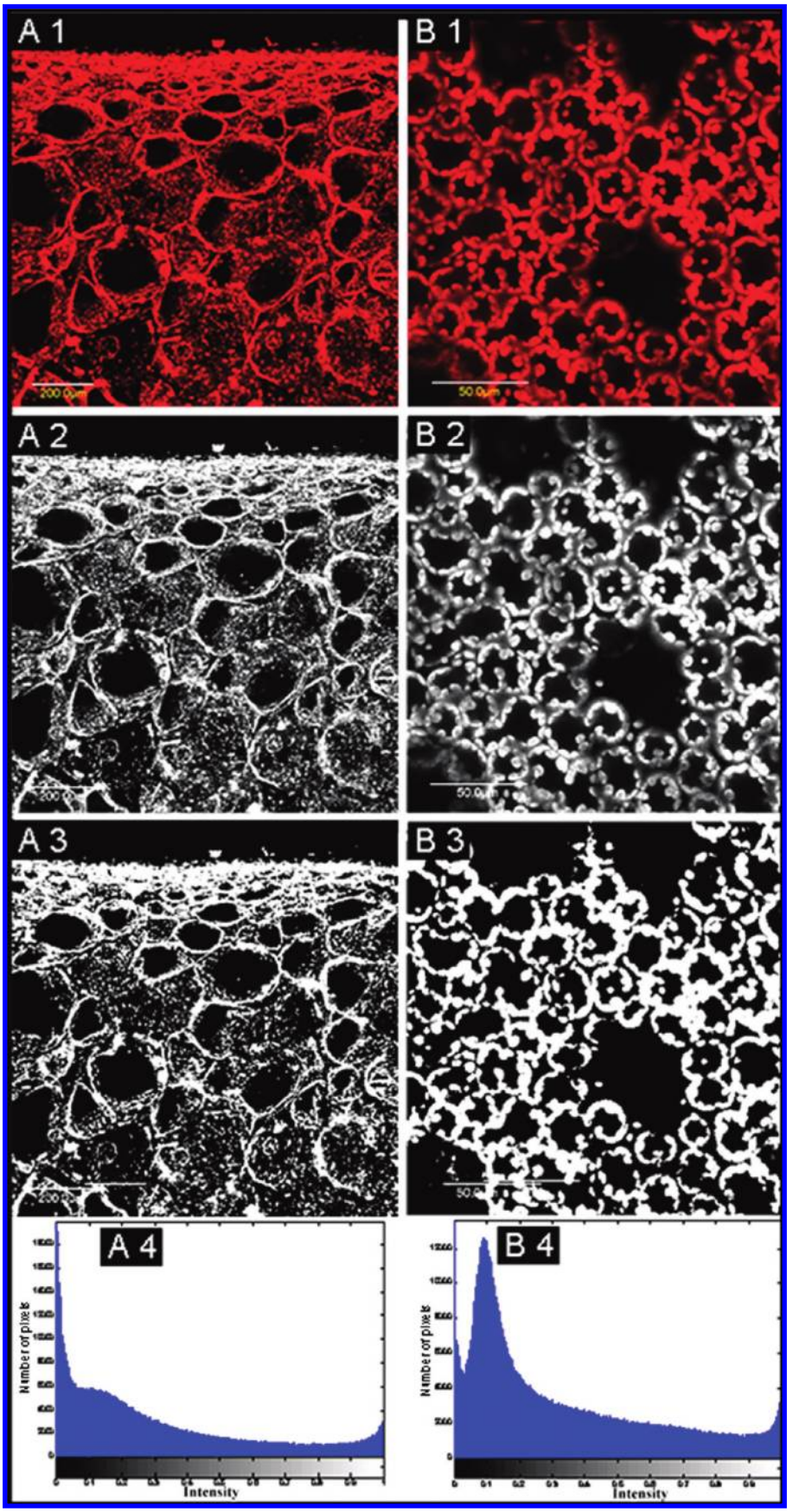

Figure 1. Confocal imaging of mature-green fruit (**A1**−**4**) and leaf (**B1**−**4**) tissues of the *IP* genotype taken with a confocal microscope: 1, transmitted light image with chlorophyll emitting red light; 2, image illustrating the saturation intensity with bright colors representing high intensity and vice versa; 3, binary image in which the white areas represent the presence and location of chlorophyll and the black areas represent parts lacking chlorophyll; 4, histogram depicting the intensity level of a saturation image with a threshold value separating chlorophyll from other parts of the image.

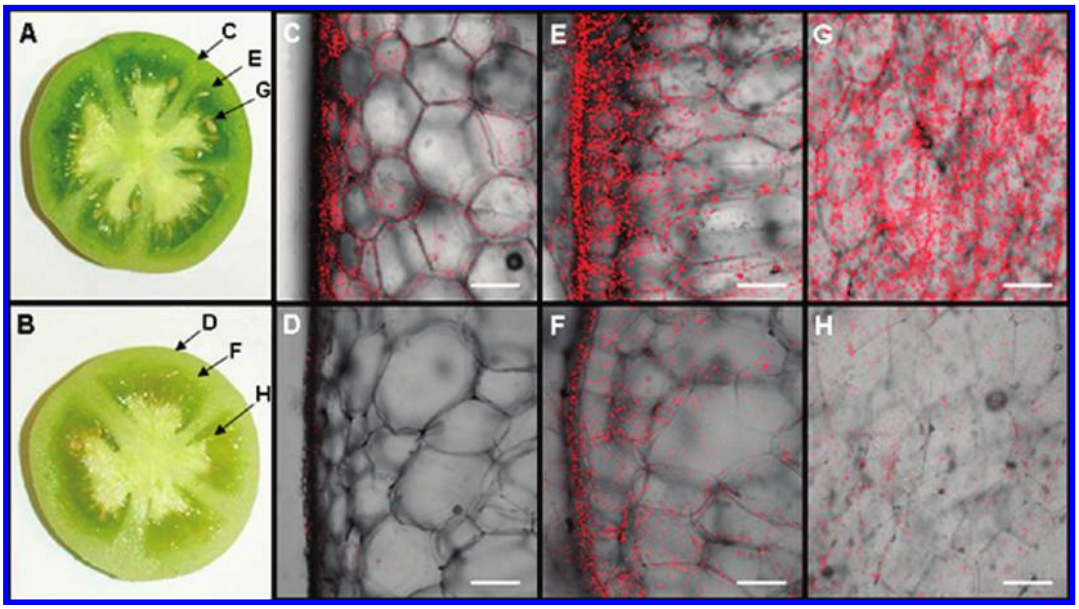

Figure 2. Representative visual display of mature-green fruits and fruit chloroplasts of the *IP* genotype in comparison to its near-isogenic control: a mature-green fruit of the *IP* genotype (**A**), chloroplasts in its outer mesocarp (**C**), inner mesocarp (**E**) and in gel tissue (**G**) are shown relative to the control genotype (**B**, **D**, **F**, and **H**, respectively). Scale bars for C−H = 200 μm.

Table 4. Analysis of Confocal Images Taken from Various Fruit Tissues and Leaves from Two Regions of *IP* Plants and Their Near-Isogenic Counterparts[a]

		fruits			leaves	
genotype	fruit tissue	area (%)	intensity level	plant region	area (%)	intensity level
IP/IP	outer mesocarp	20.4A ± 2.1	0.78A ± 0.01	mid region	17.4A ± 1.2	0.56A ± 0.01
+/+		6.8B ± 0.7	0.76B ± 0.01	mid region	9.3B ± 2.3	0.53B ± 0.01
change (%)		+200	+3		+87	+6
IP/IP	inner mesocarp	15.4A ± 1.4	0.86A ± 0.03	top region	13.5A ± 2.9	0.54A ± 0.01
+/+		17.5A ± 0.7	0.84B ± 0.04	top region	4.1B ± 0.9	0.51 B ± 0.01
change (%)		−12	+3		+230	+6
IP/IP	gel tissue	54.4A ± 3.0	0.85A ± 0.07			
+/+		30.9B ± 1.7	0.83A ± 0.04			
change (%)		+76	+2			

[a] Area, average image area occupied by chloroplasts ± SE; intensity level, average intensity of the red light emitted by the chloroplasts ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between genotypes for each trait in fruits and in leaves separately.

that in their fruits, probably due to their vital role as photoprotective agents in photosynthesis. Total carotenoid content in leaves of the *IP* plants was 22.8% higher than in leaves of their near-isogenic counterparts and was correlated to the increase observed in their chlorophyll content (26.9%); both increases support the higher chloroplast density observed in *IP* leaves. Total carotenoid content in leaves of *IP* plants, however, was not statistically different compared to their near-isogenic counterparts as was chlorophyll content, probably due to the prominent synthesis of chlorophyll relative to carotenoids in leaves of both genotypes.

All *hp* mutants, thus far studied in detail, are characterized by increased levels of lycopene as well as other carotenoids in their mature fruits, similarly to the *IP* genotype. Most *hp* mutants, however, display higher and more consistent fruit carotenoid levels compared to the *IP* genotype, ranging usually between ~160 and 320 μg g^{-1} of FW (*2*, *9*, *11*, *12*). In this respect, *hp* mutants are advantageous for breeding purposes. However, apart from a shift in sucrose accumulation at the expense of glucose and fructose observed in *hp-1* mutant (*17*), *hp* mutants are not usually characterized by higher fruit sugar or soluble solid content when compared to their respective isogenic counterparts (I.L., personal communications). The *IP* genotype, on the other hand, displays both increased fruit pigmentation and higher sugar content, suggesting that these two quality traits can be more easily combined if this genotype is used for breeding purposes whether genes controlling these two traits are genetically linked or not.

To the best of our knowledge, this paper is the first to describe an association between carotenoid levels in ripe-red fruits and higher chlorophyll concentrations, accompanied by increased chloroplast biogenesis, in leaves and mature-green fruits of the *IP* genotype. A correlation between increased pigmentation in ripe-red fruits and increased chlorophyll concentrations in leaves and mature-green fruits was previously described in *hp* mutants by us (*9*, *11*, *14*) and others (*10*, *19*). Microscopic observations of the mature-green fruits of the *hp-2^{dg}* and *hp-2^{j}* mutants revealed a

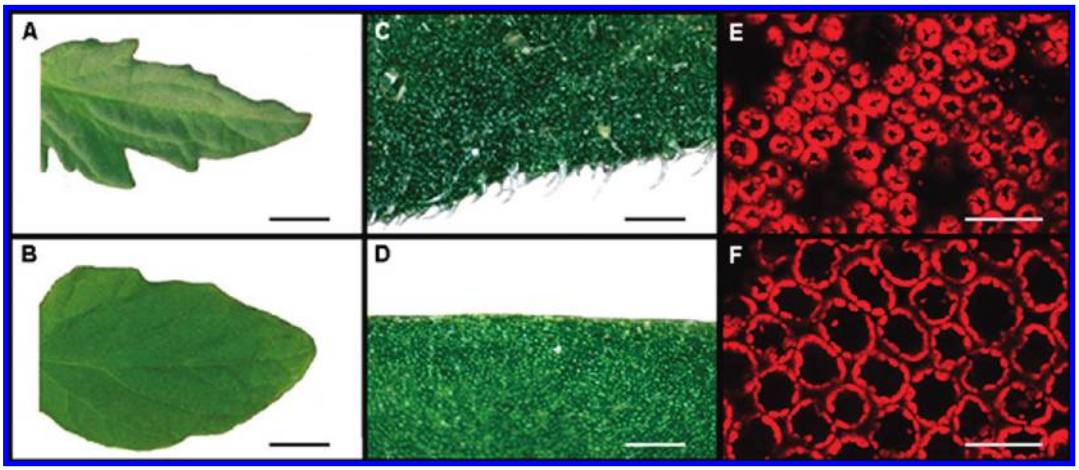

Figure 3. Representative visual display of leaves and chloroplasts in leaf mesophyll of *IP* plants in comparison to their near-isogenic controls: photo of the dorsal side of whole leaf tissue of the *IP* genotype (**A**), its enlarged view (**C**), and chloroplasts in its mesophyll cells (**E**) compared to their near-isogenic counterparts (**B**, **D**, and **F**, respectively). Scale bars: **A** and **B** = 10000 μm; **C** and **D** = 2000 μm; **E** and **F** = 50 μm.

Table 5. Chlorophyll Concentration in Mature-Green Fruits and in Leaves of *IP* Plants in Comparison to Their Near-Isogenic Counterparts[a]

	chlorophyll content (μg g^{-1} of FW)	
genotype	fruits	leaves
IP/IP	32.9A ± 2.9	2027.9A ± 65.9
+/+	25.1B ± 1.3	1598.4B ± 114.6
change (%)	+31.1	+26.9

[a] Values represent mean ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between genotypes in fruits and leaves separately.

Table 6. Levels of Selected Flavonoid Compounds in Peels of Ripe-Red Tomato Fruits of the *IP* Genotype in Comparison to Their Near-Isogenic Counterparts[a]

	genotype		
flavonoid	*IP/IP*	+/+	change (%)
quercetin	5015A ± 87	5071A ± 73	−1
naringenin flavanone	2883A ± 95	2538A ± 47	+14
kaempferol	369A ± 51	336A ± 43	+10

[a] Values represent average flavonoid concentrations (in μg g^{-1}of FW) ± SE. No significant differences were observed in levels of flavonoid compounds between genotypes.

highly significant increase in chloroplast size and number in fruit pericarp cells in comparison to their normal isogenic counterparts (*9*). Similarly, an increase in fruit chloroplast compartment size was also shown in *hp-3* mutant tomato fruits (*19*). All of these data suggest that increased plastid compartment size is a highly prevalent and a relatively successful strategy to propel increased chloroplast-accumulating metabolites, such as carotenoids, in tomato fruits. Interestingly, several attempts to genetically modify tomato carotenoids, in particular lycopene, by constitutive overexpression of genes encoding early enzymes in the pathway (the rate limiting *PHYTOENE SYNTHASE* and also *PHYTOENE DESATURASE*) met with mixed results. In some cases, transgenic tomatoes accumulated lesser total carotenoids compared to nontransformed fruits or transgenic plants became immensely dwarf in stature (*30*, *31*). Although several other transgenic approaches achieved promising results in increasing lycopene or β-carotene levels in tomato fruit (*32*−*34*), their success was limited due to the low induction folds or increases

Table 7. Effects of Various Light Conditions on Hypocotyl Length of 11-Day-Old Seedlings of the Genotype *IP* in Comparison to Their Near-Isogenic Counterparts[a]

	light conditions			
genotype	white	yellow	blue	darkness
IP/IP	6.7A ± 0.1	8.7B ± 0.2	6.0B ± 0.1	13.9B ± 0.5
+/+	6.6A ± 0.1	9.2A ± 0.2	6.6A ± 0.1	17.4A ± 0.4
change (%)	+1.5	−5	−9	−20

[a] Values represent average lengths (in cm) ± SE. Different letters indicate statistically significant differences ($P < 0.05$) between genotypes in each light condition separately.

of only one or a few carotenoid metabolites, rather than an increase in all or nearly all carotenoid metabolites as in *hp* (*9*, *12*) and *IP* (**Table 2**) genotypes. All of these experiments, however, were carried out using regular tomato genotypes for transformation. It would therefore be extremely interesting to transgenically modulate the carotenoid biosynthetic pathway in tomato genotypes with enhanced plastid biogenesis such as *hp* and *IP*.

In our study, a novel image analysis technique was applied not only on the pericarp but also on other tissues of mature-green fruits and on green leaves taken from two regions in the tomato plants. On the basis of our analyses we suggest that the higher chlorophyll concentration in mature-green fruits and in leaves of the *IP* genotype is mainly attributed to increased plastid compartment size—measured indirectly by increased tissue area occupied by chloroplasts, rather than enhanced chlorophyll biosynthesis—measured by the intensity level of our images. In this respect our results are very similar to those of previous papers displaying analysis of plastid compartment size in *hp-2^{dg}*, *hp-2^{j}*, and *hp-3* tomato mutant fruits (*9*, *19*). A more elaborated study of this phenomenon should further clarify the relationship between plastid compartment size and chlorophyll as well as carotenoid levels in the *IP* fruits. Furthermore, the relationship between plastid compartment size in mature-green and carotenoid content in ripe-red fruits is apparently due to the transformation of chloroplasts into chromoplasts during ripening (*9*, *35*). This may explain why unripe fruit with greater chloroplast biogenesis result in fruits with higher levels of carotenoids, as was demonstrated earlier in *hp-2^{dg}* and *hp-3* fruits (*9*, *19*).

Unlike other *hp* mutant fruits (*2*, *4*, *9*, *12*), no significant differences were found in the content of flavonoid compounds

in peel tissues obtained from ripe-red tomatoes harvested from *IP* and its near-isogenic counterpart. The reason for higher level of flavonoid compounds in skin tissues of *hp* mutant fruits was never thoroughly studied. It is, however, highly likely that in these mutants levels of flavonoid compounds are not directly associated with plastid biogenesis, but rather with increased transcription of genes active in the flavonoid biosynthesis pathway, particularly in response to light cues.

Hypocotyl inhibition response under red and far-red light and a preferentially enhanced response in hypocotyl anthocyanin accumulation under blue light were previously demonstrated in *IP* seedlings (*23*). A statistically significant hypocotyl inhibition response was found under blue and yellow light, no response under white light, but a novel and a more prominent response in total darkness. Tomato *hp* mutants display a pleiotropic hypocotyl inhibition response under a variety of light conditions, including red, far-red, blue, yellow, and most natural light conditions (*11*, *14*, *23*, *26*). In complete darkness, however, *hp* mutants do not usually show any particular phenotype, with the exception of the two extreme *hp* mutants, *hp-1*^{w} and *hp-2*^{j}. In these latter mutants, part of the reduced hypocotyl length of seedlings, measured at an early stage of seedling development and under any light conditions, including in complete darkness, can be attributed to the delayed germination of their seeds (*29*) (I.L., personal communications). The more prominent hypocotyl inhibition response of the *IP* genotype under complete darkness cannot be attributed to delayed germination, which had never been observed or reported in this genotype, and renders *IP* more skoto- than photo- morphogenic. The skotomorphogenic response ascribed here to the *IP* genotype can be attributed to its dominant nature. In particular, if the gene or gene product that is responsible for this mutant phenotype is downstream from photoreceptors and does not require active photoreceptor proteins for its function as tomato *DET1* (*10*), most probably the iso-phenotypic *DDB1* do. In other words, it is highly likely that *IP* causes promotion of light signaling amplification, as was suggested previously (*26*), even without the presence of active photoreceptors.

In conclusion, the *IP* genotype was found in this study to exhibit highly desirable traits such as increased levels of fruit carotenoids, including lycopene, and elevated content of soluble solids. This genotype can therefore be considered to be a valuable genotype for tomato breeders attempting to genetically introduce functional and taste qualities into tomato fruits.

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