RESEARCH PAPER

Overexpression of **UV-DAMAGED DNA BINDING PROTEIN 1** links plant development and phytonutrient accumulation in *high pigment-1* tomato

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Abstract

Fruits of tomato plants carrying the *high pigment-1* mutations *hp-1* and *hp-1m* are characterized by an increased number of plastids coupled with enhanced levels of functional metabolites. Unfortunately, *hp-1* mutant plants are also typified by light-dependent retardation in seedling and whole-plant growth and development, which limits their cultivation. These mutations were mapped to the gene encoding UV-DAMAGED DNA BINDING PROTEIN 1 (*DDB1*) and, recently, fruit-specific RNA interference studies have demonstrated an increased number of plastids and enhanced carotenoid accumulation in the transgenic tomato fruits. However, whole-plant overexpression of *DDB1*, required to substantiate its effects on seedling and plant development and to couple them with fruit phenotypes, has heretofore been unsuccessful. In this study, five transgenic lines constitutively overexpressing normal *DDB1* in *hp-1* mutant plants were analysed. Eleven-day-old seedlings, representing these lines, displayed up to 73- and 221-fold overexpression of the gene in hypocotyls and cotyledons, respectively. This overexpression resulted in statistically significant reversion to the non-mutant developmental phenotypes, including more than a full quantitative reversion. This reversion of phenotypes was generally accompanied by correlated responses in chlorophyll accumulation and altered expression of selected light signalling genes: *PHYTOCHROME A*, *CRYPTOCHROME 1*, *ELONGATED HYOCOTYL 5*, and the gene encoding *CHLOROPHYLL A/B-BINDING PROTEIN 4*. Cumulatively, these results provide the missing link between *DDB1* and its effects on tomato plant development.

Key words: *DDB1*, development, *high pigment-1*, photomorphogenesis, tomato.

Introduction

Fruits harvested from tomato plants carrying the *high pigment* (*hp*) mutations *hp-1*, *hp-1m*, *hp-2*, *hp-2*', and *hp-2** are typified by increased plastid biogenesis, coupled with enhanced levels of functional metabolites, including carotenoids and flavonoids (Bino *et al.*, 2005; Kolotilin *et al.*, 2007; Azari *et al.*, 2010). These mutant plants also share significant photomorphogenic responses at the seedling stage followed by retarded growth and development, which limit their cultivation (Mochizuki Kamimura, 1984; Levin *et al.*, 2003; Lieberman *et al.*, 2004).

The photomorphogenic nature of *hp-2*, *hp-2*', and *hp-2** mutants underlies the identification of *DEETIOLATED1* (*DET1*; Chory *et al.*, 1989), encoding a negative regulator of photomorphogenesis, as the gene causing these mutant phenotypes (Mustilli *et al.*, 1999; Levin *et al.*, 2003). To confirm this gene’s identification, Davuluri *et al.* (2004) overexpressed several versions of the tomato *DET1*, under constitutive and fruit-specific promoters, in normal tomato genotypes. Their results were inconsistent: only 25% of the transgenic lines displayed phenotypes deviating from the non-transgenic controls. All visible phenotypes were invariably characteristic of exaggerated light sensitivity, mainly shorter bushy plants with dark-green immature fruits, reminiscent of *hp* mutants. Immature fruits harvested from these transgenic lines displayed higher chlorophyll levels, and, upon ripening, 2- and 5-fold higher levels of...
lycopene and β-carotene, respectively. These phenotypes were associated with a reduction in DET1 expression, showing that reduced expression levels of DET1 in tomato fruits can cause increased levels of chlorophylls and carotenoids. However, no quantitative results were presented with respect to seedling or plant development in association with DET1 transcript levels.

To harness the positive effects of DET1 suppression in tomato fruits without the negative effects on plant growth, Davuluri et al. (2005) inhibited DET1 mRNA accumulation using RNA interference (RNAi) constructs driven by fruit-specific promoters. Fruits harvested from the resulting transgenic plants displayed significantly higher levels of both carotenoids and flavonoids, similar to hp mutant fruits (Bino et al., 2005; Azari et al., 2010), whereas other parameters of plant stature and fruit quality remained unchanged.

Using the candidate gene approach it was also found that hp-1 and hp-1* plants are characterized by two different mutations in the gene encoding the evolutionarily conserved protein UV-DAMAGED DNA BINDING PROTEIN 1 (DDB1), shown to interact with DET1 (Schroeder et al., 2002; Lieberman et al., 2004; Liu et al., 2004). To confirm this gene’s identification, Wang et al. (2008) used fruit-specific RNAi constructs to show that a reduced amount of DDB1 transcript in tomato fruits is associated with an increased number of plastids and enhanced carotenoid accumulation.

Efforts constitutively to complement hp-1 mutant phenotypes with the normal tomato DDB1, or to obtain constitutive silencing of this gene, failed to yield viable plants, suggesting that these manipulations are lethal to tomato plants (Liu et al., 2004; Wang et al., 2008). In contrast, transgenic tomato plants ectopically overexpressing DDB1 in three genetic backgrounds have recently been obtained. Three groups of phenotypes were observed among these transgenic plants (Azari et al., 2010): (i) plants characterized by significantly reduced levels of DDB1 transcript in fruits and mature vegetative tissues but not in seedlings or during early plant development; (ii) plants with significantly reduced fruit number, fruit weight, and seed number per fruit associated with increased DNA methylation, recapernating the link between DDB1 and chromatin biology (O’Connell and Harper, 2007); and (iii) a reversion of phenotype in hp-1 mutant plants accompanied by additional novel phenotypes. An analysis of the transgenic plants of the first group has already been presented (Azari et al., 2010), and the study of plants belonging to the second group will be presented elsewhere. The analysis of transgenic plants forming the third group is presented herein, focusing on seedling development under skotomorphogenic and photomorphogenic conditions, and on early plant development under natural light conditions.

The results demonstrate a clear reversion of phenotype in the hp-1 mutant seedlings overexpressing the non-mutant DDB1, establishing the link between this gene and its photomorphogenic and developmental effects during seedling and early plant growth. This link is important because it is also obscured in the model plant Arabidopsis thaliana, the first plant species analysed for the effects of DDB1 on seedling development. Unlike tomato, which carries a single copy of DDB1 (Lieberman et al., 2004), A. thaliana has two DDB1 homologues, DDB1A and DDB1B. Whereas A. thaliana ddb1b mutants are lethal, single mutant ddb1a plants show no visible phenotype but can enhance the photomorphogenic responses attributed to det1 in det1 ddb1a double-mutant seedlings (Schroeder et al., 2002). Therefore, this is the first study to establish a direct link between DDB1 and seedling photomorphogenesis, as well as early plant development.

Materials and methods

Plant material and experimental set-up

Seeds from the normal open-pollinated tomato (Solanum lycopersicum) cv. Ailsa Craig (AC), AC +/-, and a line nearly isogenic and homozygous for the hp-1 mutation, AC hp-1hp-1, were obtained as previously described (Lieberman et al., 2004). Five independent AC hp-1hp-1 T0 transgenic plants, carrying the non-mutant DDB1 under the control of the 35S promoter from cauliflower mosaic virus (CaMV) for whole-plant constitutive expression, were generated. These transgenic plants were identified by polymerase chain reaction (PCR) with primers specific to the DDB1 transgene, and were self-pollinated. At each successive generation, seeds acquired from 32 self-pollinated transgenic plants of each of these five lines were germinated and genotyped again. At the end of this process, five stable T5 lines resulting from T3 lines that did not segregate for the transgene were obtained (termed DDB113–DDB117). Five azygous (null) hp-1 hp-1 control plants (AZ hp-1hp-1), which do not carry the transgene, were selected from each line at the T1 generation and advanced by self-pollination. One of these lines was randomly chosen to represent the azygous control line in the present experiments, the other control lines being normal AC +/- and mutant AC hp-1hp-1.

The seedling experiment was carried out in an environmentally controlled growth chamber (16 h light/8 h dark) at 25±1°C. Two blocks were used in a randomized block design, with 20 seedlings per plot comprised of 240 ml pots filled with planting soil (Shaham Givat Ada Ltd, Givat Ada, Israel). During the experiment, seeds of the five transgenic lines as well as the three control lines were allowed to germinate and grow for 11 d under two photomorphogenic conditions, i.e. white light and under a yellow optical screen, and under skotomorphogenic conditions (complete darkness). The yellow screen prevented the transmission of light below a wavelength of 500 nm (Fig. 1). Germination and seedling growth under these broad-range red light conditions has been shown to result in an exaggeration of the differences in hypocotyl development between hp mutants and normal seedlings (Mochizuki and Kamimura, 1984; Adamse et al., 1989; Peters et al., 1989, 1992; Kerckhoffs et al., 1997). The light intensities used in this experiment in relation to the spectral wavelengths are presented in Fig. 1 and were measured with an Apogee pyranometer CS-300L (Campbell Scientific, North Logan, UT, USA).

For the greenhouse experiment, seeds of the five transgenic lines were sown on 6 April 2009 and allowed to germinate and grow under natural light conditions until 11 May 2009. They were then transplanted into 8.0 l pots in the greenhouse, arranged as a randomized three block design, with eight plants of each genotype per block. A minimal temperature of 18°C was maintained in this greenhouse with no supplemental light.
Plasmid construction and plant transformation

The expression cassette was constructed in pBINPLUS (van Engelen et al., 1995). This vector contains a marker gene encoding neomycin phosphotransferase II resistance driven by the CaMV 35S promoter (Benley and Chua, 1990). An additional 35S promoter is located upstream of a cloning site for the insertion of genes of interest. The full-length normal tomato DDB1, excluding its untranslated regions (GenBank accession no. AY452480), was amplified by the proofreading Pwo DNA polymerase (Genaxis Biotechnology, Kronberg/Taunus, Germany) from young leaves of the open-pollinated tomato cv. AC and cloned into the pGEM-T Easy vector system (Promega Corporation, Madison, WI, USA). A sequence-validated DDB1 clone was subcloned into pBluescript KS (+) (Stratagene, Agilent Technologies, La Jolla, CA, USA) following digestion with EcoRI. This clone was finally inserted, as a SalI–SpeI fragment forming a transcriptional fusion between the 35S promoter and the nos terminator, into pBINPLUS. The transgenic tomato plants were generated by Agrobacterium tumefaciens-mediated transformation of cotyledons from 10-d-old seedlings (McCormick, 1991).

Transgenic characterization of plants

To validate incorporation of the transgene into the plant genome, genomic DNA was extracted from young leaves of individual transformed plants according to Fulton et al. (1995). These DNA samples served as templates in PCRs with primers complementary to the CaMV 35S promoter and the DDB1 gene: 5′-CCTTCGCAAGACCCTTCCTCTA-3′ and 5′-TTCCITTCAGTGGGCC TITGTTATCAA-3′. The PCRs were performed in a T-GRADIENT thermal cycler (Biometra, Analytik Jena, Gottingen, Germany) in a volume of 25 μl containing 15 ng of template DNA, 10 pmol of each primer, 0.2 mM of each dNTP, 2 mM MgCl₂, 0.5 U of Taq DNA polymerase, and 1× PCR buffer. The PCR consisted of an initial incubation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, and annealing at 58 °C for 40 s and 72 °C for 40 s. Final elongation, at 72 °C, was carried out for 5 min. PCR products were visualized by electrophoresis in 1.0% agarose gels stained with ethidium bromide.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from hypocotyls and cotyledons harvested from 11-d-old seedlings of the five transgenic and three control lines as previously described (Kolotilin et al., 2007). Prior to cDNA synthesis, PCR was carried out with each of the extracted RNA samples as templates and selected primers to validate that these RNA samples were free of DNA. cDNA synthesis was carried out using a Superscript II synthesis kit (Invitrogen, Carlsbad, CA, USA). Gene-specific primers were designed using Primer Express Software Version 3.0 (Applied Biosystems, Warrington, UK). qRT-PCRs were performed using a SYBR Green qPCR DyNAmo Flash kit (Finzymes, Espoo, Finland) on an ABI PRISM 7000 cycler (Applied Biosystems). The cycling conditions were 7 min at 95 °C, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. The genes and forward and reverse primers used in the qRT-PCRs were (5′→3′): DDB1, CCCTCCCTTGAAGTGGTTGGT and CTTGAACTGATGTGCATGAC; PHOTOTRICHOME A (PHYA), GATTTCATGACCTCCTTAAAGGC; and CRYPTOCRHOME 1 (CRY1), GAGGTACGATGACCAAGCC and CGCATCCAAGATGACAG. The gene encoding CHLOROPHYLL A/B-BINDING PROTEIN 4 (CAB4), AAAAAATTCATGCACTGAG and CACCTCGGGTAGATCCGTGTC.

Results

Overexpression of normal DDB1 in hp-1 seedlings

Five independent and stable T₃ transgenic hp-1 tomato lines constitutively overexpressing the normal tomato DDB1 were generated. Seeds representing each of these lines were germinated and grown for 11 d under white light, a yellow plastic screen, or in complete darkness. At the end of the experiment, cDNA synthesized from total RNA samples, extracted separately from hypocotyls and cotyledons of seedlings representing these transgenic lines, was subjected to transcript level analysis of total DDB1 and compared with the three non-transgenic controls (AC +/+ , AC hp-1/hp-1, AC hp-1/hp-1, hp-1/hp-1).
and AZ hp-1/hp-1). Because no transcriptional or phenotypic differences were obtained between the two hp-1 control lines, their results were combined prior to the final analyses and designated AC hp-1/hp-1. Results presented in Table 1 show that although DDB1 transcript levels varied among the five transgenic lines, in general they displayed statistically significant overexpression of the gene in both hypocotyls (up to ~73-fold) and cotyledons (up to ~221-fold) compared with the hp-1 control lines under the three light treatments. The only exceptions were the cotyledons of DDB113, DDB115, and DDB117 seedlings grown under skotomorphogenic conditions (total darkness). Although cotyledons of these lines displayed a 3- to 4-fold increase in DDB1 transcript compared with the hp-1 control line under these conditions (Table 1), the elevated transcript levels were found to be statistically insignificant. These results, particularly those obtained under photomorphotic conditions, indicated that the expression of the non-mutant DDB1 had been successfully up-regulated. In addition, no statistically significant differences were obtained between the average transcript levels of DDB1 in both hypocotyls and cotyledons of the hp-1 lines and their normal counterparts under any of the three light conditions. These latter results confirmed that DDB1 is not transcriptionally modulated in seedlings of the mutant hp-1 lines compared with their normal controls under photo- or skotomorphogenic conditions.

Overexpressing normal DDB1 in hp-1 plants reverses their developmental phenotypes

A representative 11-d-old seedling of each of the transgenic and non-transgenic lines, germinated and grown under the three light conditions, is presented in Fig. 2. This figure demonstrates the reversion of phenotype obtained in the transgenic lines, which was visually more explicit under the two photomorphogenic conditions. Analysis of hypocotyl length showed that the seedlings of all five transgenic lines shared statistically significantly longer hypocotyls than the hp-1 control lines under white light and the yellow screen (Table 2). However, this apparent reversion of phenotype appeared to be incomplete because all transgenic lines were characterized by reduced average hypocotyl length compared with the normal control line (AC+/+). Under the yellow screen, these reductions were statistically significant, but under white light only two lines, DDB113 and DDB115, showed significantly reduced average hypocotyl length compared with the normal control line. In total darkness, the differences in average hypocotyl length among lines were markedly decreased. This reduced effect of the transgene could be attributed to the much lower up-regulation of DDB1 obtained in total darkness compared with the other two light conditions (Table 1), also indicating that the 35S promoter is directly or indirectly affected by light. Although in total darkness, all transgenic lines displayed longer average hypocotyl length compared with the hp-1 genotypes (Table 2), in two of the transgenic lines, DDB116 and DDB117, this effect was not statistically significant. On the other hand, the average hypocotyl length of two transgenic lines, DDB114 and DDB115, was not statistically different from that of the normal isogenic line (AC+/+). This indicated that a reversion of phenotype in hypocotyl development was also obtained in total darkness and that the reduced hypocotyl length of the hp-1 line under skotomorphogenic conditions could be attributed to the action of the mutant DDB1 gene.

The effect of the transgene on cotyledon weight was less consistent than its effect on hypocotyl length (Table 2). Under white light, for example, relatively small and statistically insignificant differences were obtained in average cotyledon weight between most lines examined in this study, including the control lines. The only exception was DDB113, which displayed a significantly reduced average cotyledon weight compared with all other lines. Under the yellow screen, on the other hand, all five transgenic lines displayed significantly reduced average cotyledon weight compared with both the normal and hp-1 control lines. The latter displayed significantly reduced average cotyledon weight compared with the normal isogenic control (Table 2). Because the average cotyledon weight of the five transgenic

Table 1. Relative transcript levels of DDB1 in hypocotyls and cotyledons of 11-d-old seedlings under three light conditions

| Genotype     | Hypocotyls |           |           | Cotyledons |           |           |
|--------------|------------|-----------|-----------|------------|-----------|-----------|
|              | Darkness   | Yellow screen | White light | Darkness   | Yellow screen | White light |
| DDB113       | −5.8±0.4 a (6.0) | −3.8±0.0 a (49.4) | −2.4±0.0 a (13.5) | −4.1±0.2 ab (3.7) | −4.1±0.5 b (36.6) | −2.3±0.1 a (10.0) |
| DDB114       | −5.2±0.3 a (11.0) | −3.6±0.1 a (60.3) | −1.7±0.2 a (27.1) | −2.5±0.5 a (18.2) | −2.7±0.5 a (148.4) | −2.7±0.6 a (6.7) |
| DDB115       | −5.1±0.4 a (12.2) | −3.9±0.3 a (44.7) | −2.8±0.3 a (9.0) | −4.0±0.6 a (4.0) | −2.3±0.5 a (221.4) | −1.6±0.2 a (20.1) |
| DDB116       | −5.1±0.6 a (12.2) | −3.6±0.2 a (60.3) | −1.7±0.2 a (27.1) | −3.6±0.3 a (6.0) | −2.6±0.6 a (164.0) | −1.8±0.1 a (16.4) |
| DDB117       | −5.4±0.6 a (9.0) | −3.4±0.2 a (73.4) | −1.5±0.51 a (33.1) | −4.3±0.5 ab (3.0) | −2.9±0.3 a (121.5) | −1.8±0.0 a (16.4) |
| AC+/+        | −8.3±0.4 b (0.5) | −6.9±0.6 b (22.2) | −5.1±0.44 b (0.9) | −6.3±0.4 c (0.4) | −7.6±0.9 c (1.1) | −4.9±0.1 b (0.7) |
| AC hp-1/hp-1 | −7.6±0.3 b (1.0) | −7.7±0.4 b (1.0) | −5.0±0.25 b (1.0) | −5.4±0.2 bc (1.0) | −7.7±0.5 c (1.0) | −4.6±0.2 b (1.0) |
| PIF          | 2.4×10^{-7} | 2.1×10^{-8} | 3.6×10^{-8} | 2.9×10^{-5} | 1.1×10^{-10} | 4.6×10^{-10} |

AC+/+, normal AC seedlings; AC hp-1/hp-1, pooled homozygous and azygous hp-1 mutant seedlings in the AC background; DDB113-DDB117, hp-1/hp-1 mutant seedlings overexpressing the normal DDB1 under the CaMV 35S promoter.

Values are displayed as average natural logarithm of transcript ±SE; in parentheses, fold change in average DDB1 transcript levels of each genotype relative to the hp-1/hp-1 genotype within each light condition.

Different lower case letters indicate statistically significant differences between genotypes within each light condition.
lines was significantly lower than that of hp-1 controls; these results implied that under yellow light, overexpression of DDB1 significantly exaggerates the hp-1 phenotype rather than reverting it, contrary to the results obtained for hypocotyl length. Interestingly, differences in average cotyledon weight among the lines following growth in total darkness were generally opposite to, and more variable than those obtained under the yellow screen. First, average cotyledon weight of the hp-1 control lines was significantly lower than that of the hp-1 genotypes, indicative of a reversion of phenotype. Finally, the transgenic line DDB117 displayed an average cotyledon weight which was significantly lower than that of the normal control line, indicating that in this genotype, more than full reversion of the cotyledon weight phenotype occurred in total darkness.

The results to this point indicated that overexpression of the non-mutant DDB1 affects the developmental phenotypes of hp-1 seedlings, with a significant overall tendency toward reversion to the normal phenotype. However, the reversion and its extent were both organ specific and light dependent. To substantiate these results further, the ratios of hypocotyl length as well as cotyledon weight between seedlings developing under photomorphogenic conditions (under white light or the yellow screen) and those developing under skotomorphogenic (total darkness) conditions were analysed in each of the transgenic and control lines. This analysis was expected to remove the confounding effects, such as seed quality variation among the lines, and to reveal the underlying net photomorphogenic response. The results presented in Table 3 show that following adjustment to skotomorphogenic conditions, most transgenic lines displayed both a full quantitative and statistical reversion of phenotype in their hypocotyl length. The only exceptions were lines DDB113 and DDB115 which displayed a full statistical but not quantitative reversion under white light. A similar analysis carried out on cotyledon weight was much more indicative of a reversion of phenotype than the

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**Table 2.** Average hypocotyl length and cotyledon weight of 11-d-old seedlings grown under the three different light conditions

| Genotype | Hypocotyl length (cm) | Cotyledon weight (mg) |
|----------|-----------------------|-----------------------|
|          | Darkness | Yellow screen | White light | Darkness | Yellow screen | White light |
| DDB113   | 14.6±0.3 b | 8.6±0.1 b | 6.4±0.1 b | 2.6±0.1 cd | 13.0±0.4 c | 31.7±1.0 b |
| DDB114   | 15.3±0.4 ab | 8.6±0.1 b | 7.2±0.1 a | 3.6±0.2 bc | 14.0±0.4 c | 30.5±1.2 a |
| DDB115   | 15.2±0.3 ab | 8.7±0.1 b | 6.5±0.1 b | 3.2±0.2 c | 13.9±0.4 c | 30.2±1.3 a |
| DDB116   | 13.7±0.5 bc | 8.5±0.1 b | 7.0±0.1 a | 4.5±0.3 ab | 13.1±0.4 c | 40.2±1.2 a |
| DDB117   | 14.1±0.5 bc | 8.4±0.1 b | 7.0±0.1 a | 1.9±0.2 d | 13.9±0.4 c | 41.0±1.1 a |
| AC +/-   | 16.2±0.3 a | 9.4±0.1 a | 7.4±0.1 a | 3.2±0.2 c | 21.0±0.9 a | 43.6±1.2 a |
| AC hp-1/hp-1 | 12.6±0.2 c | 3.6±0.1 c | 4.4±0.1 c | 4.9±0.2 a | 18.2±0.4 c | 40.1±1.2 a |
| PRF      | 1.6×10^{-10} | 1.7×10^{-124} | 7.6×10^{-74} | 5.5×10^{-19} | 9.0×10^{-40} | 3.9×10^{-8} |

AC +/-, normal AC seedlings; AC hp-1/hp-1, pooled homozygous and azygous hp-1 mutant seedlings in the AC background; DDB113–DDB117, T₃ hp-1/hp-1 mutant seedlings overexpressing the normal DDB1 under the CaMV 35S promoter. Values are displayed as mean ±SE. Different lower case letters indicate statistically significant differences between genotypes within each light condition.
results presented in Table 2. First, under the yellow screen, most lines displayed reversion to some extent, with one line, DDB117, displaying a full quantitative and statistical reversion of phenotype. Secondly, under white light, most lines, i.e. DDB113, DDB114, DDB115, and DDB117, displayed an average ratio which was significantly higher than that obtained for the hp-1 control lines. Again, line DDB117 displayed an average ratio that was significantly higher than even that of the normal control line, indicating more than full reversion of phenotype (Table 3).

To explore further the growth characteristics of the transgenic lines as compared with the controls, seeds of each line were germinated and grown in a nursery for 30 d under natural light conditions. These seedlings were then transplanted in the greenhouse to follow their growth phenotypes. Stem length was recorded on the day of transplantation, and at 14, 22, 29, and 50 d post-transplantation. On the day of transplantation, all of the transgenic lines already displayed an average stem length that significantly exceeded that of the hp-1 lines; moreover, two of the lines, DDB114 and DDB115, were significantly more developed than the non-mutant control line (Table 4). A similar developmental phenotype was obtained 50 d post-transplantation, with two lines, DDB115 and DDB116, each exhibiting an average stem length that even exceeded that of the control lines (Table 4). In addition, the average growth rate of all of the transgenic lines was significantly higher than that of the hp-1 control lines, and one line, DDB116, exhibited a growth rate that significantly exceeded even that of the normal control line (Table 4). It is worth noting that irrespective of their growth performance, none of the transgenic plants displayed the typical hypersensitive characteristics of the hp-1 control lines, i.e. leaf-curled, reduced internode length, and anthocyanin accumulation in the vegetative tissues and fruit shoulders (data not shown).

### Table 3. Photomorphogenic to skotomorphogenic ratios of hypocotyl length and cotyledon weight of 11-d-old seedlings

| Genotype     | Hypocotyl length ratio | Cotyledon weight ratio |
|--------------|------------------------|------------------------|
|              | Yellow/darkness | White/darkness | Yellow/darkness | White/darkness |
| DDB113       | 0.6±0.0 a     | 0.4±0.0 a     | 5.0±0.1 bc     | 12.2±0.4 bc    |
| DDB114       | 0.6±0.0 a     | 0.5±0.0 a     | 3.9±0.1 cd     | 11.0±0.3 c     |
| DDB115       | 0.6±0.0 a     | 0.4±0.0 a     | 4.3±0.1 bc     | 12.2±0.4 bc    |
| DDB116       | 0.6±0.0 a     | 0.5±0.0 a     | 2.9±0.1 e      | 8.8±0.3 d      |
| DDB117       | 0.6±0.0 a     | 0.5±0.0 a     | 7.2±0.2 a      | 21.3±0.5 a     |
| AC +/-       | 0.6±0.0 a     | 0.5±0.0 a     | 7.2±0.3 a      | 13.6±0.4 b     |
| AC hp-1/hp-1 | 0.3±0.0 b     | 0.3±0.0 b     | 3.7±0.1 d      | 8.1±0.2 d      |
| P(f)         | 6.0×10^{-10} | 5.9×10^{-5}   | 9.8×10^{-7}   | 2.0×10^{-7}   |

AC +/-, normal AC seedlings; AC hp-1/hp-1, pooled homozygous and azygous hp-1 mutant seedlings in the AC background; DDB113–DDB117, T3 hp-1/hp-1 mutant seedlings overexpressing the normal DDB1. Values are displayed as mean ± SE. Different lower case letters indicate statistically significant differences between genotypes within each light condition.

### Table 4. Growth performance of plants grown under natural light conditions

| Genotype     | Stem length (cm) at | Growth rate (cm d⁻¹) |
|--------------|---------------------|----------------------|
|              | Transplantation | 50 d post-transplantation |
| DDB113       | 7.7±0.2 c    | 122.7±2.9 c       | 2.3±0.1 c    |
| DDB114       | 8.9±0.2 a    | 146.0±3.8 ab     | 2.7±0.1 ab   |
| DDB115       | 8.9±0.2 a    | 151.0±2.7 a      | 2.8±0.0 ab   |
| DDB116       | 8.6±0.2 ab   | 155.0±3.3 a      | 2.9±0.1 a    |
| DDB117       | 8.3±0.2 abc  | 145.7±3.9 ab     | 2.7±0.1 ab   |
| AC +/-       | 7.9±0.1 bc   | 138.0±3.9 b      | 2.6±0.1 b    |
| AC hp-1/hp-1 | 6.8±0.2 d    | 109.0±2.0 d      | 2.0±0.0 d    |
| P(f)         | 1.6×10⁻¹⁰ | 1.4×10⁻²⁰        | 2.7×10⁻‘²²   |

AC +/-, normal AC seedlings; AC hp-1/hp-1, pooled homozygous and azygous hp-1 mutant seedlings in the AC background; DDB113–DDB117, T3 hp-1/hp-1 mutant seedlings overexpressing the normal DDB1 under the CaMV 35S promoter. Values are displayed as mean ± SE; in parentheses. Different lower case letters indicate statistically significant differences between genotypes within each light condition.
yellow screen and under white light conditions. Overexpression of DDB1 in the hp-1 lines led to a statistically significant reduction in chlorophyll concentration, to levels that were not statistically different from those of the normal control lines, indicating a reversion of phenotype. This decrease in chlorophyll concentration was statistically more pronounced under the yellow plastic screen \[P(F)=2.7\times10^{-3}\] compared with white light conditions \[P(F)=6.0\times10^{-14}\]. No significant response was obtained in complete darkness \[P(F)>0.05\]. In fact, under the latter conditions, only minute and nearly undetectable levels of chlorophyll were found in all lines studied. These traces may represent background, chlorophyll precursors, or other metabolites with absorbance similar to that of chlorophylls.

Although hp-1 mutant plants are usually known for their increased chlorophyll accumulation in immature-green fruits and mature leaves (Cookson et al., 2003; Caspi et al., 2008), a significantly lower chlorophyll concentration was observed in cotyledons taken from hp-1 control lines relative to their normal isogenic controls grown under white light and yellow screen, and, surprisingly, in complete darkness as well (Table 5). Following overexpression of DDB1, higher average chlorophyll concentrations were obtained in cotyledons of the five transgenic hp-1 lines grown under the three light conditions. This effect was much more prominent under the two photomorphogenic treatments, where cotyledons of the transgenic lines displayed average chlorophyll contents that were quantitatively, and in most cases significantly, also higher than those of the normal control line. These results were strongly indicative of a reversion of phenotype obtained by overexpression of the normal DDB1 in hp-1 mutant plants.

Overexpressing normal DDB1 in hp-1 seedlings alters transcript levels of light signalling genes

Based on the results to this point, it could be concluded that DDB1 significantly affects developmental as well as metabolomic processes in developing seedlings and young plants, and that these effects are light and organ dependent. Therefore, transcript levels of selected light-regulated genes were analysed separately in the hypocotyls and cotyledons of seedlings representing the transgenic lines and compared with their normal controls under the three illumination conditions. In addition to DDB1, four light signalling genes (Quail, 2002) were examined: two photoreceptor genes, PHYA and CRY1, a downstream effector gene encoding a bZIP transcription factor, HY5, and a light-regulated structural gene, CAB4. To simplify the presentation, all five transgenic lines and the two hp-1 controls were separately pooled prior to the final analysis. The analysis of the data, generally displayed in Fig. 3, indicated the following. (i) With the exception of PHYA in hypocotyls and generally as expected, the light treatment had a strong and highly significant effect on the transcript levels of the analysed genes, substantiating the choice of genes \[P(F)\] values for light treatment in the hypocotyls were \[4.9\times10^{-24}, 5.0\times10^{-1}, 1.2\times10^{-9}, 1.9\times10^{-4}\], and \[1.6\times10^{-24}\], while in the cotyledons they were \[1.6\times10^{-8}, 9.0\times10^{-38}, 3.3\times10^{-7}, 7.9\times10^{-7}\], and \[9.0\times10^{-7}\], for DDB1, PHYA, CRY1, HY5, and CAB4, respectively. (ii) Similarly to DDB1, no significant transcriptional differences were generally found between the two hp-1 lines and their normal counterpart in either hypocotyls or cotyledons within each light treatment. The exceptions in this respect were the transcriptional up-regulation of HY5 in cotyledons under yellow light (Fig. 3I) and of CAB4 in hypocotyls under complete darkness (Fig. 3E) in the hp-1 genotypes. Interestingly, the latter gene was significantly down-regulated in hypocotyls of the hp-1 genotypes grown under the yellow screen (Fig. 3E). (iii) Contrary to the results obtained in the comparison between hp-1 and normal genotypes, wider transcriptional effects were observed in the five transgenic lines (Fig. 3). These effects were light dependent; in some cases they were organ specific and in a few others they were opposite to the DDB1 transcript levels.

Several transcriptional effects of particular interest were identified in the transgenic lines. (i) Under certain illumination conditions, overexpression of DDB1 resulted in up-regulation of PHYA and CRY1 transcript levels: these genes initiate light signalling and are therefore upstream of DDB1.

### Table 5. Chlorophyll concentration in hypocotyls and cotyledons of 11-d-old seedlings under different light conditions

| Genotype | Hypocotyls | Cotyledons |
|----------|------------|------------|
|          | Darkness | Yellow screen | White light | Darkness | Yellow screen | White light |
| DDB113   | 1.2±0.2 a | 48.4±1.4 b | 77.8±5.1 b | 78.0±8.7 a | 705.5±53.5 a | 1034.8±13.6 ab |
| DDB114   | 0.6±0.1 a | 48.8±0.7 b | 67.8±1.5 b | 56.8±8.1 abc | 632.7±17.2 a | 1080.4±6.7 a |
| DDB115   | 0.8±0.1 a | 43.5±0.7 b | 71.5±2.5 b | 40.5±2.3 bc | 639.2±3.9 a | 1102.8±9.9 a |
| DDB116   | 1.1±0.3 a | 47.6±1.0 b | 68.9±2.4 b | 60.7±13.4 abc | 643.5±11.1 a | 903.0±24.6 b |
| DDB117   | 1.2±0.1 a | 44.5±1.9 b | 75.2±2.1 b | 69.8±8.7 ab | 629.0±8.9 a | 1050.4±17.1 ab |
| AC +/+   | 1.1±0.1 a | 47.1±1.3 b | 65.2±2.5 b | 63.1±6.1 ab | 529.6±23.5 b | 994.6±23.5 b |
| AC hp-1/hp-1 | 1.3±0.1 a | 85.2±2.7 a | 109.0±2.7 a | 37.6±2.9 c | 437.2±9.5 c | 774±14.9 c |
| P(F)     | 0.06      | 2.7×10^{-2} | 6.0×10^{-14} | 1.7×10^{-4} | 4.7×10^{-12} | 2.3×10^{-18} |

AC+/+, normal AC seedlings; AC hp-1/hp-1, pooled homozygous and azygous hp-1 mutant seedlings in the AC background; DDB113-DDB117, T3 hp-1/hp-1 mutant seedlings overexpressing the normal DDB1 under the CaMV 35S promoter. Values are displayed as average chlorophyll concentration (mg/g FW) ±SE. Different lower case letters indicate statistically significant differences between genotypes within each light condition.
Fig. 3. Transcript level analysis of DDB1, PHYA, CRY1, HY5, and CAB4 in hypocotyls (A, B, C, D, and E, respectively) and cotyledons (F, G, H, I, and J, respectively) of 11-d-old transgenic hp-1 tomato seedlings overexpressing normal DDB1 in comparison with their normal and mutant control lines under different light treatments (black bars, pooled hp-1 mutant and azygous control lines; white bars, non-mutant line; grey bars, pooled T3 hp-1 mutant seedlings overexpressing normal DDB1 under the CaMV 35S promoter); different letters below the bars indicate statistically significant differences between genotypes within each gene and light condition.
These effects were highly consistent under the yellow screen, slightly less apparent under total darkness, but completely missing under white light in both hypocotyls and cotyledons (Fig. 3B, C, G, and H). (ii) Under the yellow screen, the downstream effector HY5, as well as the downstream structural CAB4 gene, were similarly and significantly affected. Interestingly, the effect of DDB1 on CAB4 transcript levels in hypocotyls was reversed in the dark (Fig. 3E).

**Discussion**

Tomato plants carrying the hp mutations are characterized by increased plastid compartment size in their developing fruits. This increase is coupled with enhanced levels of functional metabolites in their mature ripe-red fruits: carotenoids, primarily lycopenes, flavonoids, and vitamins C and E (Bino et al., 2005; Kolotilin et al., 2007). Due to their positive effect on fruit lycopene content, these mutations were introgressed into elite processing tomato cultivars for its cost-effective extraction as a natural food supplement and colorant (Levin et al., 2006; Levin, 2009). However, seedlings carrying these mutations also display negative photomorphogenic responses manifested by slower growth and development under virtually all light conditions, but deviating more significantly from their normal counterparts under red and far-red light conditions (Mochizuki and Kamimura, 1984; Peters et al., 1989; Kerckhoffs et al., 1997; Levin et al., 2003; Lieberman et al., 2004). Mature mutant plants also display retarded growth and development which is exaggerated under higher light intensities and coupled with reduced agricultural performance (Davuluri et al., 2004; Levin, 2009). These negative phenotypes restrict the agricultural utilization of hp mutant plants, particularly under extreme light intensities, and a frequently asked question is whether these light-dependent negative developmental phenotypes are pleiotropic manifestations of the same gene responsible for the beneficial fruit phenotypes or caused by other genetically linked genes.

The photomorphogenic response of hp-2, hp-2^l, and hp-2^dg mutant seedlings (Mochizuki and Kamimura, 1984; Adamse et al., 1989; Peters et al., 1989, 1992; Kerckhoffs et al., 1997) was the basis for the suggestion that DET1, encoding a negative regulator of photomorphogenesis in *A. thaliana* (Chory et al., 1989), underlies these mutant phenotypes (Mustilli et al., 1999; Levin et al., 2003). This gene’s identification was surprising because: (i) the effect of det1 mutants on phytonutrient content had never been noticed in *A. thaliana* fruits, probably due to their dehiscent nature (Azari et al., 2010); and (ii) the photomorphogenic response of hp-2, hp-2^l, and hp-2^dg mutant tomato seedlings to diverse light conditions was different from those displayed by det1 mutants in *A. thaliana*: whereas *A. thaliana* det1 mutations are epistatic to phytochrome genes and display a strong de-etiolated phenotype under total darkness, the manifestation of the tomato hp-2, hp-2^l, and hp-2^dg mutants is highly dependent upon the presence of active phytochromes and they therefore do not display substantial de-etiolated phenotypes in the dark (Mustilli et al., 1999; Fig. 1). However, Davuluri et al. (2004) and, in particular, Davuluri et al. (2005) produced highly convincing evidence that suppressed DET1 expression can lead to significantly increased levels of both carotenoids and flavonoids, similar to hp mutant fruits (Bino et al., 2005; Azari et al., 2010). Although a portion of the transgenic lines obtained by Davuluri et al. (2004) displayed a shorter, bushy plant stature, no quantitative results were presented to associate seedling or plant development with DET1 transcript levels. The candidate gene approach was also used to show that hp-1, and hp-1^w mutant plants are typified by two different point mutations in the gene encoding an evolutionarily conserved protein homologous to human and *A. thaliana* DDB1. This approach was based on the genetic interaction of DDB1 with DET1, as well as the biochemical interaction of its protein product with DET1 (Schroeder et al., 2002; Lieberman et al., 2004; Liu et al., 2004). As recently summarized (Azari et al., 2010), these interactions have since been elaborated in *A. thaliana*, tomato, and humans, positioning DDB1 as an excellent candidate gene responsible for the hp-1, hp-1^w mutant phenotypes.

To confirm that modulation of tomato DDB1 expression can indeed result in phenotypes characteristic of hp-1 and hp-1^w mutant plants, Wang et al. (2008) used fruit-specific promoters combined with RNAi to show that reduced DDB1 transcript levels in tomato fruits increase the number of plastids and enhance carotenoid accumulation in ripe-red transgenic tomato fruits relative to their non-transgenic controls. These results showed that, similar to DET1, modulated DDB1 expression can lead to increased levels of functional metabolites in tomato fruits as in hp-1 and hp-1^w mutant plants. However, the link between the developmental phenotypes of the transgenic seedlings and DDB1 expression could not have been established in that study because transgene expression was fruit specific.

The link between DDB1 and plant developmental phenotypes is particularly difficult to determine because: (i) DDB1 is localized to the centromere (Lieberman et al., 2004), a chromosomal region of exceptionally low recombination frequency, and it is therefore much less accessible to genetic dissection; and (ii) mutations in either of the two *A. thaliana* homologues, DDB1A or DDB1B, do not produce photomorphogenic phenotypes on their own (Schroeder et al., 2002). This link could be established through complementation of hp mutant phenotypes by overexpressing the normal tomato DDB1 cDNA under the 35S promoter or through constitutive silencing of DDB1 in normal tomato plants. However, prior to the present study, efforts to obtain such transgenic plants were unsuccessful (Liu et al., 2004; Wang et al., 2008).

The present results demonstrate a reversion of the developmental phenotypes in hp-1 mutant seedlings overexpressing the non-mutant DDB1. This reversion of phenotype extends to the mature plant, resulting in enhanced plant growth and development. Thus a link is established, for the first time, between DDB1 and its photomorphogenic
and developmental effects during early plant growth, a link which was obscured in the model plant *A. thaliana* (Schroeder et al., 2002). The results also complement the finding of Wang et al. (2008) of a reduced *DDB1* transcript level in ripe-red tomato fruits increasing the number of plastids and enhancing carotenoid accumulation. As such, the present results reveal the missing link between plant development and phytonutrient accumulation in fruits of *hp*-1 tomato mutants. The reversion of phenotype in terms of chlorophyll accumulation, which was obtained separately in both hypocotyls and cotyledons, also illustrates the effect of *DDB1* on the plant’s metabolite network. Of particular note is the opposite nature of chlorophyll accumulation in *hp*-1 cotyledons and hypocotyls in comparison with their normal controls (Table 5): it suggests that whole seedlings may be subjected to confounding effects, thus hindering accurate interpretations. In view of this phenomenon, results from studies using whole seedlings to explore responses of tomato genotypes to varying light conditions warrant a second look.

The present results also show that the developmental reversion of phenotypes obtained in seedlings is also associated with transcriptional regulation of light signalling genes which are both up- and downstream of *DET1*, and possibly *DDB1*, while the mutant affects downstream genes only. Although these effects were most strongly manifested under the yellow screen, which greatly affected possible *DDB1* transcript levels (Table 1), they demonstrate the involvement of *DDB1* in their transcriptional regulation, and confirm that tomato *DDB1* is an integral part of the light signalling machinery.

To the best of our knowledge, the link established in this study between plant development and phytonutrient accumulation in fruits of *hp* tomato mutants has never been presented elsewhere; moreover, it suggests that the negative pleiotropic developmental effects of *hp* mutant plants can only be avoided by: (i) transgenic means, as has been demonstrated previously in tomato (Davuluri et al., 2005; Wang et al., 2008); (ii) identification of novel tomato *DDB1* or *DET1* mutants with lesser effects on whole plant stature; and, finally (iii) extragenic means, as has been demonstrated in *A. thaliana* (Hu et al., 2002).

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