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The tomato *dark green* mutation is a novel allele of the tomato homolog of the *DEETIOLATED1* gene

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Abstract A comprehensive, multi-generation, allele test, carried out in this study, suggests that the tomato mutations *dark-green* (*dg*) and *high pigment* 2^j (*hp-2*^{j}) are allelic. The *hp-2*^{j} mutant is caused by a mutation in the tomato homolog of the *DEETIOLATED1* (*DET1*) gene, involved in the signal transduction cascade of light perception and morphogenesis. This suggestion is in agreement with the exaggerated photomorphogenic de-etiolation response of homozygous *dg* mutants grown under modulated light conditions. Sequence analysis of the *DET1* gene was carried out in *dg* mutants representing two different lines, and revealed a single A-to-T base transversion in the second exon of the *DET1* gene in comparison with the normal wild-type sequence. This transversion results in a conserved Asparagine^{34}-to-Isoleucine^{34} amino-acid substitution, and eliminates a recognition site for the *Acl*I restriction endonuclease, present in the wild-type and in the other currently known tomato mutants at the *DET1* locus. This polymorphism was used to develop a PCR-based DNA marker, which enables an early genotypic selection for breeding lycopene-rich tomatoes. Using this marker and sequence analysis we demonstrate that an identical base transversion also exists in *dg* mutants of the cultivar Manapal, in which the natural *dg* mutation was originally discovered. A linkage analysis, carried out in a F_2 population, shows a very strong linkage association between the *DET1* locus of *dg* mutant plants and the photomorphogenic response of the seedlings, measured as hypocotyl length ($12 < \text{LOD Score} < 13$, $R^2 = 51.1\%$). The results presented in this study strongly support the hypothesis that the tomato *dg* mutation is a novel allele of the tomato homolog of the *DET1* gene.

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Introduction

Plants respond to light intensity, direction, duration and spectral quality by modulating their developmental processes in an array of interactions that are referred to as photomorphogenesis. Photomorphogenic mutants have proven to be an excellent tool in the study of the complex interactions between light and plant development, and some of them have also been harnessed in several breeding programs of agricultural crops. Photomorphogenic mutants have been reported in a number of species, including *Arabidopsis, Sorghum, Brassica*, tobacco, tomato and pea. In general, these mutants may be classified either as defective in photoreceptors, or altered in some element of the light signal-transduction chain (Chory 1993).

Several light-hypersensitive mutants have been described in tomato (*Lycopersicon esculentum*). Among these, mutants carrying the monogenic recessive *high pigment* (*hp-1* and *hp-2*) and *dark green* (*dg*) mutations are characterized by their exaggerated light responsiveness. Seedlings of such homozygous mutant plants display higher anthocyanin levels and shorter hypocotyls in comparison to their semi-isogenic wild-type plants or heterozygous counterparts (Mochizuki and Kamimura 1984). These homozygous mutants are usually also characterized by higher fruit and foliage pigmentation (Wann et al. 1985). The high fruit pigmentation of these mutants is due to significantly elevated levels of carotenoids, primarily lycopene, and flavonoids in the mature ripe-red fruit. Due to their effect on fruit color, attributed by a higher lycopene content, *hp* and *dg* mutations have been introgressed into several commercial processing and fresh-market tomato cultivars that are currently marketed as Lycopene Rich Tomatoes (LRT) (Wann 1997).

The *hp-1* mutant was originally discovered as a spontaneous mutant in 1917 at the Campbell Soup Company

farms (Riverton, N.J.) (Reynard 1956), the *hp-2* mutant was reported in the Italian San Marzano variety in 1975 (Soressi 1975) and the *dg* mutant appeared in trellised planting of the Manapal variety (Konsler 1973). Despite some initial confusion, it is now clear that *hp-1* and *hp-2* mutations map to the tomato chromosomes 2 and 1, respectively, and are therefore non-allelic (Van Tuinen et al. 1997; Yen et al. 1997). At each of these loci, two mutant alleles have been identified: *hp-1* and *hp-1*^{w}, *hp-2* and *hp-2*^{j} (Kerckhoff and Kendrick 1997; Van Tuinen et al. 1997). In a recent study, the *HP-2* gene was cloned and found to encode the tomato homolog of the *Arabidopsis* nuclear protein *DEETIOLATED1* (*DET1*) (Mustilli et al. 1999). In the more phenotypically extreme *hp-2*^{j} mutation, a C-to-T transition was found in exon 11, which gave rise to the substitution of a conserved proline for a serine residue in the C-terminal region of the DET1 protein. In the *hp-2* mutant, it was suggested that an A-to-T transversion directs an alternative splicing of intron 10, leading to a nine-base deletion in exon 11, and resulting in a deletion of the first three amino acids encoded by this exon. The *hp-2* deletion is located within the second putative NLS presumably resulting in mislocalization of the protein. The milder phenotypic display by the *hp-2* mutation might be due to its "leakiness", as about 10% of the normal DET1 protein is produced in the mutant plants (Mustilli et al. 1999).

The *dg* mutant is phenotypically similar to other *hp* mutants, but has a much darker mature-green fruit, resulting from a higher total chlorophyll content (Konsler 1973; Wann et al. 1985). The identity as well as the map location of the *DG* gene was unknown prior to this report. In this study, we provide experimental data suggesting that *dg* is an alternative allele at the *HP-2* locus, identified as the tomato *DET1* gene. This suggestion is based on a detailed allele test, linkage analysis, sequence analyses of the tomato *DET1* gene cloned from *dg* mutant plants, and DNA polymorphism as well as a comparative sequence analysis between the *dg* cv Manapal mutant and its corresponding isogenic wild-type plants. In addition, this study provides a polymorphic PCR-based DNA marker that may be used for marker-assisted selection of *dg* mutant plants at the early seedling stage.

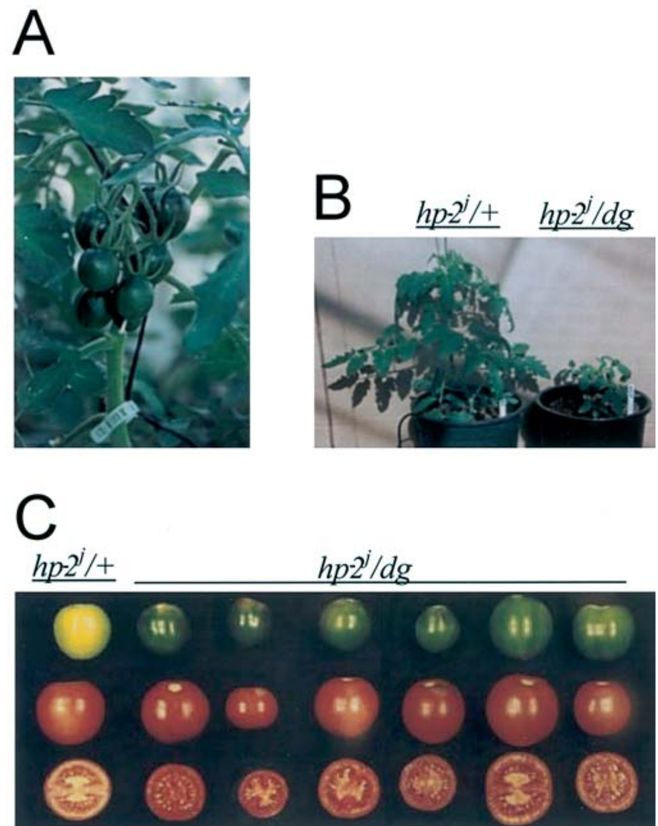

Fig. 1A–C Visual phenotypic characterization of heterozygous *hp-2*^{j}/*dg* F_1 plants. **A** A typical heterozygous *hp-2*^{j}/*dg* F_1 plant with mature-green fruits; **B** Developmental differences between heterozygous *hp-2*^{j}/+ and *hp-2*^{j}/*dg* F_1 plants, 2 months after sowing; **C** Mature-green and ripe-red fruits of heterozygous *hp-2*^{j}/+ and six of the seven heterozygous *hp-2*^{j}/*dg* F_1 plants

Materials and methods

Plant material

The *hp-2* and *hp-2*^{j} mutants and the corresponding semi-isogenic wild-type tomato seeds (*L. esculentum* cv Money Maker) were kindly provided by R.E. Kendrick and M. Koornneef (Wageningen Agriculture University, The Netherlands). The *dg* mutant and its corresponding isogenic wild-type tomato seeds (*L. esculentum* cv Manapal) were provided by R.T. Chetelat (Tomato Genetics Cooperative, UC Davis, USA). Seeds from wild-type tomato (*L. esculentum* cv Ailsa Craig) and a line nearly isogenic and homozygous for the *hp-1* mutation were obtained from J.J. Giovannoni (Texas A and M University, USA). F_2 seeds of a cross between determinate *dg* mutant plants and wild-type plants (cv PETO4303) were obtained from E. Peleg (Hazera Genetics seed company, Israel). Lines n474 and n935, that were used to sequence the entire *DET1* gene, are *dg* mutant lines developed by the late R. Frankel, D. Lapushner and I. Levin at the Volcani Center, Israel. Several wild-type tomato cultivars used in this study, such as Ailsa-Craig, M82, VF-36, 189, 124, 139, NC8288 and Florida, were from seed stocks available at the Volcani center.

Crosses, growth conditions and experimental design

An initial cross was carried out between *hp-2*^{j} and *dg* mutant plants during the summer of 2000 in a screen-house at the Volcani Center, Bet Dagan, Israel. In this cross, the indeterminate *hp-2*^{j} (cv Money Maker) plants served as a maternal line, whereas a mixture of pollen, derived from ten unrelated determinate *dg* parental breeding lines, represented the paternal genotype. Seven of the F_1 plants resulting from the above cross were planted in an environmentally controlled greenhouse at the Volcani Center during the winter of 2000/2001. All of these plants displayed a delayed growth development, and set mature-green and ripe-red fruits with a characteristic dark-green phenotype identical to homozygous *dg*/*dg* plants (Fig. 1). All of these F_1 plants were found to be true hybrids, using markers complementary to the *SELF-PRUNING* locus (Pnuelli et al. 1998). F_2 seeds were individually extracted from each one of these plants at the end of the season.

Sixty four F_2 seeds, from each one of the seven original F_1 plants, were germinated under a yellow plastic screen, omitting transmittance of light spectra under 500 nm (Mochizuki and Kamimura 1984), in the Spring of 2001 in an environmentally controlled greenhouse at a commercial nursery (Hish-Shtill, Nehalim, Israel). The

photomorphogenic response of seedling hypocotyl length was visually compared to several wild-type genotypes germinated under the same conditions. Twenty eight of the more etiolated F_2 seedlings, representing each one of the seven original F_1 plants (a total of 196 plants), were then planted in an open field at the Hula valley research station located at northern Israel.

DNA was extracted from five plants chosen at random from each one of the seven F_2 populations (a total of 35 plants). These DNA samples were genotyped using a DNA marker developed based on a mutation discovered, in this research, in the tomato *DET1* homolog of *dg* mutants. Lycopene was extracted from ripe-red fruits, sampled from 21 of these genotyped plants, and its content was determined. Fourteen plants that set an insufficient amount of fruits were discarded from further analysis.

F_3 Seeds were extracted individually from each one of the above F_2 plants. A sample of 20 F_3 seeds, derived from six heterozygous F_2 plants (*dg*/*hp-2^j*), representing six of the seven original F_1 plants (a total of 120 seeds) were sampled. These seeds were germinated under a yellow plastic screen in an environmentally controlled growth chamber (26 °C day/18 °C night) during the autumn of 2001. The hypocotyl-length of the resulting seedlings was individually measured after 7 days, and compared to the length of wild-type seedlings.

Linkage analysis

A linkage-analysis study between the tomato *DET1* locus and the exaggerated photomorphogenic de-etiolation response characterizing *dg* mutants, was carried out using F_2 seeds of a cross between determinate *dg* mutant plants and wild-type plants (cv PETO4303). These seeds were germinated under a yellow plastic screen in an environmentally controlled growth chamber during the autumn of 2001 (26 °C day/18 °C night). The hypocotyl-length of 86 individual F_2 seedlings was measured 9 days after sowing and their genotype determined using the DNA marker developed in this study.

Genomic DNA extraction

Genomic DNA was extracted from individual plants. The extraction procedure was according to Fulton et al. (1995).

PCR primers

Sequence analysis and locus-specific primer design were carried out with the DNAMAN, Sequence Analysis Software version 4.1 (Lynnon BioSoft, Quebec, Canada). All DNA primers used during the course of this study were purchased from M.B.C Molecular Biology Center Ltd., Ness-Ziona, Israel. PCR primers used to clone overlapping fragments spanning the entire coding sequence of the tomato *DET1* gene were:

TDR1: 5′-GTA CAC CTT AGT TGC TCG AGG GCG TG-3′
CRISR: 5′-GTG ATT TCT AGG TTG ATT TCA ATC TAG AG-3′
TDF2: 5′-GAT CCT AAT TCG AGC CCT CCT T-3′
TDF1: 5′-GGA TGG AGC TAT ACT TGA CGA AAG GG-3′
TDR: 5′-GCC GTT GCT TTA TAC CGC TCA GGA AA-3′
TDR2: 5′-CAC TAG CAT CTA CGG GTC TGT TAT G-3′.

PCR primers used to amplify the tomato *DET1* genomic DNA fragment flanking the *dg* mutation site were:

TD-M2 F1: 5′-TTC TTC GGA TTG TCC ATG GT-3′
TD-M2 R1: 5′-CAC CAA TGC TAT GTG CCA AA-3′.

PCR reaction

The amplification reactions (25 µl final volume) were performed with 10 ng of template DNA, 25 mM of TAPS (pH = 9.3 at 25 °C), 50 mM of KCl, 2 mM of $MgCl_2$, 1 mM of B-mercaptoethanol, 0.2 mM of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 10 ng of each of two primers and 1 unit of thermostable *Taq* DNA polymerase (SuperNova *Taq* polymerase, Madi Ltd., Rishon Le Zion, Israel). Reactions were carried out in an automated thermocycler (MJ Research Inc., Watertown, Mass., USA). Initial incubation was at 94 °C for 1 min, followed by 34 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and polymerization at 72 °C for 1.5 min. Final polymerization at 72 °C was carried out, for 3 min, after the above cycles have been completed. The PCR amplification products were visualized by electrophoresis in 1.0% agarose gels and detected by staining with ethidium bromide.

Cloning and sequencing of the tomato *DET1* cDNA from *dg* mutant plants

Total RNA was extracted from breaker fruits (200 mg of fresh weight) of individual *dg* mutant plants representing two independent lycopene-rich tomato breeding lines, n474 and n935, and the open-pollinated wild-type cultivars Ailsa-Craig and Money Maker. The RNA extraction was employed using the TRIzol reagent system (GibcoBRL Life Technologies, Gaithersburg, Md., USA). Total RNA was used as a template for first-strand cDNA synthesis using the Superscript pre-amplification system (GibcoBRL Life Technologies, Md., USA). The cDNA prepared was used as a template, in a PCR reaction, to amplify three overlapping fragments of the gene encoding the tomato *DET1* from the two *dg* mutant plants. A fragment, 1,783-bp in length, was amplified in two sequential PCR reactions. The first reaction was primed with a TDF2-TDR2 primer combination. The resulting DNA product, 1,816-bp in length, was excised from an agarose gel, purified using the GENECLEAN II kit (BIO 101 Inc., La Jolla, Calif., USA), and used for a nested PCR reaction primed with TDF2-CRISR. The two other fragments were amplified directly from the cDNA template using the primer combinations TDF2-TDR and TDF1-CRISR. The three PCR products were then cloned into the pGEM-T Easy vector using the pGEM-T Easy Vector Systems according to the manufacturer's recommendations (Promega Corporation, Madison, Wis., USA). Three independent clones of each of the three amplified fragments were sequenced, based on the vector T7, SP6, and the tomato *DET1* complementary primers (TDF2, TDF1 and TDR1), using an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, Calif., USA). Equivalent fragments were cloned and sequenced from the wild-type cv Ailsa Craig and cv Money Maker. To confirm that the mutation discovered also exists in *dg* mutant plants of the cultivar Manapal, in which the *dg* mutation was originally discovered, we also cloned and sequence analyzed genomic DNA flanked by primers TD-M2 F1 and TD-M2 R1 of *dg* and wild-type isogenic cv Manapal plants.

Lycopene extraction and analytical determination

Lycopene was extracted from pericarp tissue of fresh ripe-red fruits. Pericarp tissue of a sample of 20 fruits per individual plant was minced to puree in a blender. An aliquot of 2 g of puree was taken from each sample and stirred with 40 ml of extraction buffer for 30 min in the dark. The extraction buffer consisted of n-hexane:isopropanol:acetone (2:1:1) and contained 0.05% BHT. Phase separation was achieved by the addition of 20 ml of 0.1 M NaCl. The organic upper-phase was collected for analysis. Spectrophotometric absorbance at 472 nm was used to calculate the lycopene concentration using E1% = 3,450 (Davis 1965).

Statistical analyses

Analyses of variance were carried out with the JMP Statistical Discovery software (SAS Institute Inc., Cary, N.C., USA). Linkage analysis and LOD score determination were carried out using QGENE software version 3.06d (Nelson 1997). Alignment of amino-acid sequences was carried out using the Clustal method (Higgins and Sharp 1988).

Results

Allele test of *dg* and *hp-2^j* mutants

Seven F_1 hybrid *hp-2^j/dg* plants initially indicated that *dg* and *hp-2^j* are allelic. All of these F_1 plants set fruit that turn extremely green at the mature-green fruit developmental stage, and exhibited delayed growth performances in comparison to heterozygous *hp-2^j/+* plants (Fig. 1). Such extreme greening is a characteristic phenotype of *dg* tomato mutants and appears much darker than *hp-2^j* mutants (data not shown). These seven plants were confirmed as true hybrids using a PCR genotyping protocol based on a tomato *SELF-PRUNING* (*SP*) gene sequence (Pnuelli et al. 1998). All seven F_1 plants were found to be heterozygous at the *SP* locus as would be expected in this cross where an indeterminate *hp-2^j/hp-2^j* maternal line was crossed with a determinate *dg/dg* paternal line.

To extend the analysis to the F_2 generation, seeds were individually extracted from each of the seven hybrid plants and a sample of 64 seeds, from each plant, was germinated under a yellow plastic screen that eliminates spectral transmittance of a light wave-length below 500 nm. Although variable, all of these F_2 seedlings displayed a divergent hypersensitive photomorphogenic phenotype, visualized as much shorter hypocotyl lengths, in comparison to wild-type lines germinated under the same conditions (i.e. cv Money Maker and M82; data not shown).

To validate the allelic relationship between *dg* and *hp-2^j*, we analyzed adult F_2 plants as well. Twenty eight of the more-etiolated F_2 seedlings, representing each one of the seven original F_1 plants (a total of 196 plants), were planted in an open field. Five plants from each of these seven F_2 populations were chosen at random for genotyping and fruit lycopene analysis (a total of 35 plants). Genotyping the *DET1* locus in these plants (see below) was carried out using DNA samples extracted from true leaves of each individual plant. Ripe-red fruits of 21 plants were analyzed for lycopene content. The fruit lycopene content of these F_2 plants exceeded those of their wild-type controls (Table 1). No *hp-2^j/hp-2^j* genotype was found among the plants participated in this analysis, because we initially selected the more-etiolated plants for further analysis (see above). The *hp-2^j/hp-2^j* genotype most probably failed to enter this selection because of the extremely slow seedling development, short hypocotyls and the severe de-etiolated phenotypes displayed by such genotypes. The hypocotyl-length photomorphogenic response of the *hp-2^j/hp-2^j* mutant, in comparison to other hypersensitive mutants of tomato, is presented in Table 2.

The allelic test between *dg* and *hp-2^j* was finalized by hypocotyl-length photomorphogenic response analysis of F_3 seedlings. F_3 seeds representing heterozygous *hp-2^j/dg* plants from six of the seven F_2 populations were collected separately and were germinated in a temperature-controlled growth chamber under a yellow plastic screen. The hypocotyl-length of single plants was measured 7 days after sowing. Results demonstrate that all of the segregating F_3 *hp-2^j/dg* seedlings were hypersensitive-photomorphogenic as compared to the wild-type controls (Table 3).

Table 1 Lycopene content in mature red fruits of parental lines and randomly chosen F_2 plants. Different superscript letters represent statistically significant differences between genotypic means ($P < 0.05$), based on the Tukey-Kramer HSD test (Kramer 1956)

Genotype	Line	N	Lycopene ± SE (ppm)	Lycopene range (ppm)
+/+	M82	6	$72^{B} \pm 2$	64–78
+/+	Money maker	2	$83^{B} \pm 5$	79–88
hp-2^j/hp-2^j	Money maker	2	$171^{A} \pm 25$	146–197
dg/dg	LRT915	1	245^{A}	–
dg/dg	F_2	6	$224^{A} \pm 13$	182–263
hp-2^j/dg	F_2	15	$193^{A} \pm 10$	121–262

Table 2 Hypocotyl-length photomorphogenic response of tomato wild-type and mutant seedlings at the *DET1* and *HP-1* loci. Seedlings were grown under a yellow plastic screen for 7 days after sowing. Different superscript letters represent statistically significant differences between means ($P < 0.05$), within each genetic background, based on the Tukey-Kramer HSD test (Kramer 1956)

Genotype	Genetic background	N	Hypocotyl length (mean ± SE)
+/+	Money maker	39	$6.4^{A} \pm 0.2$
hp-2/hp-2	Money maker	38	$5.7^{B} \pm 0.1$
hp-2^j/hp-2^j	Money maker	14	$2.5^{C} \pm 0.3$
+/+	Ailsa Craig	40	$7.5^{A} \pm 0.1$
hp-1/hp-1	Ailsa Craig	40	$4.7^{B} \pm 0.1$
+/+	M82	73	$7.5^{A} \pm 0.1$
hp-1/hp-1	M82	40	$5.0^{B} \pm 0.1$
dg/dg	M82	40	$4.9^{B} \pm 0.2$

Table 3 Hypocotyl-length photomorphogenic response of wild-type and segregating F_3 *hp-2^j/dg* seedlings. Seedlings were grown under a yellow plastic screen for 7 days after sowing. Different superscript letters represent statistically significant differences between means ($P < 0.05$) based on the Tukey-Kramer HSD test (Kramer 1956)

Genotype	N	Line	Hypocotyl length (cm)	
			Mean ± SE	Range
+/+	18	Money maker	$5.6^{A} \pm 0.1$	4.5–6.6
Segregating *hp-2^j/dg*	62	F_3	$2.8^{B} \pm 0.1$	1.2–4.4

Sequence characterization of the tomato *DET1* in *dg* mutants

Two independently developed lycopene-rich breeding lines, homozygous for the *dg* mutation, were initially used for sequence analysis. We thoroughly sequenced the entire coding region of the tomato *DET1* cDNA and a portion of its 5′ (50 bp) and 3′ (336 bp) untranslated

regions. A single transversion of Thymine-to-Adenine was observed when the *dg* mutant sequence was compared to wild-type plants. This single-base transversion was located to nucleotide 29 of the second exon of the *DET1* gene sequence (Fig. 2), and thus its deduced amino-acid substitution would be Asparagine^{34}-to-Isoleucine^{34}. This mutation also renders the *dg* mutant allele uncleavable by the *Acl*I restriction endonuclease. Sequence analysis carried out on genomic DNA cloned from *dg* and wild-type plants of the cultivar Manapal confirmed that the single-base transversion that differentiates these two genotypes is identical to the transversion revealed in the aforementioned analyses.

The *dg* mutation can be identified as a cleaved amplified polymorphic DNA sequence

We developed a DNA marker to identify *dg* mutant plants based on the sequence results (Fig. 2). The PCR primers (TD-M2 F1 and TD-M2 R1) were designed to amplify the genomic DNA sequences flanking the *dg* mutation. These genomic sequences were amplified, cleaved by *Acl*I and revealed a clear polymorphism between *dg* and several wild-type plants representing nine lines or cultivars (Money Maker, Ailsa Craig, M82, VF-36, 189, 124, 139, NC8288 and Florida; data not shown). However, to substantiate these results, we genotyped the *DET1* locus in *dg* mutant plants of the Manapal cultivar (LA3005 and LA2451) and compared them to their fully isogenic wild-type plants (LA3007). The polymorphism obtained (Fig. 3A), substantiates our hypothesis that *dg* is a mutation at the tomato homolog of *DET1* gene. The segregation of individual F_2 plants using the DNA marker developed is presented in Fig. 3B.

Linkage analysis between the *DET1* locus and the photomorphogenic response

A linkage analysis study was carried out to test the association between the *DET1* locus and the characteristic hypersensitive-photomorphogenic response displayed by *dg* mutant seedlings (i.e. inhibition of the hypocotyl-elongation phenotype). For this purpose, F_2 seeds of a cross between determinate *dg* mutant plants and wild-type plants (cultivar PETO4303) were germinated under a yellow plastic screen in a controlled growth chamber. Nine days after sowing, the hypocotyl-length of individual seedlings was recorded and their *DET1* locus was genotyped using the DNA marker described above. The results demonstrate a clear association between the *DET1* locus and hypocotyl-length (Table 4). Homozygous recessive *dg*/*dg* seedlings displayed a highly significant inhibition of hypocotyl elongation, indicative of a more-exaggerated photomorphogenic de-etiolation response, in comparison to the two other genotypic groups (12 < LOD Score < 13, R^2 = 51.1%). These results confirm that the mutation identified in the *DET1* locus of *dg*

A. Nucleotide

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w.t. I. ATGTTCAAAACTAACAATGTTACCGCCAGGCTTTTTGAGCGCCAGATTTGCACCCCTGCTCC
dg   I. ATGTTCAAAACTAACAATGTTACCGCCAGGCTTTTTGAGCGCCAGATTTGCACCCCTGCTCC
        **************************************************************

w.t. TGGCACCAGC II. ATCCATCGTGCCAGAAGATTTTATGAGAACGTTGTACCAAGTTATACCAT
dg   TGGCACCAGC II. ATCCATCGTGCCAGAAGATTTTATGAGATCGTTGTACCAAGTTATACCAT
     **********     **************************** *********************

w.t. ATACGATGTTGAATGTCCCGACCATTCATTTCGCAAGTTCACGGATGACGGT
dg   ATACGATGTTGAATGTCCCGACCATTCATTTCGCAAGTTCACGGATGACGGT
     ****************************************************
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B. Amino Acid

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w.t. I. MFKTNNVTARLFERQICTPAPGTS II. IHRARRFYENVVPSYTIYDVECPDHSFRKFTDDG
dg   I. MFKTNNVTARLFERQICTPAPGTS II. IHRARRFYEIVVPSYTIYDVECPDHSFRKFTDDG
        ************************     ********* ************************
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Fig. 2A, B Alignment of nucleotide (**A**) and deduced amino-acid sequence (**B**) of the first (I) and part of the second (II) exons of the *DET1* gene of normal wild-type (w.t.) and homozygous *dg* plants. The site of the mutation, in both the nucleotide and amino-acid sequences, is indicated by an *enlarged bold letter*. The *Acl*I cleavage recognition sequence is *underlined* in the wild-type nucleotide sequence

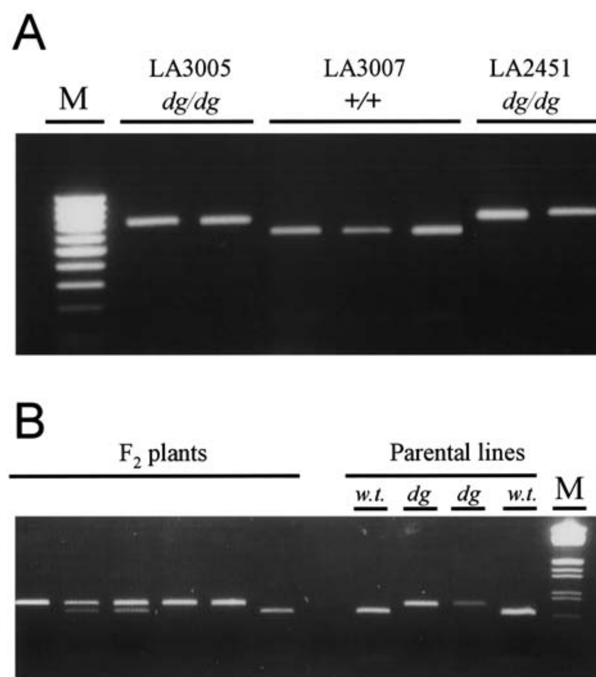

Fig. 3A, B Cleaved amplified polymorphic DNA marker to identify *dg* mutant plants. **A** Polymorphism obtained in a cv Manapal background (M = GeneRuler 100-bp DNA ladder); **B** Segregation in the F_2 population (M = Lambda DNA/*Eco*RI + *Hin*dIII)

Table 4 Linkage analysis between the tomato *DET1* locus and the hypocotyl-length photomorphogenic response. Seedlings were grown under a yellow plastic screen for 9 days after sowing. Different superscript letters represent statistically significant differences between means (P < 0.05) based on the Tukey-Kramer HSD test (Kramer 1956)

Genotype	N	Hypocotyl length ± SE (cm)	LOD score	R^2
+/+	18	$7.7^A \pm 0.3$	12 < LOD < 13	51.1%
dg/+	35	$7.7^A \pm 0.2$		
dg/*dg*	33	$5.4^B \pm 0.2$		

mutant plants is associated with one of its main characteristic phenotypes, i.e. inhibited hypocotyl elongation of seedlings.

Discussion

Tomato cultivars carrying the *high pigment* (*hp*) and/or the *dark green* (*dg*) mutations represent a unique, non-GMO platform for the production of health-promoting functional-food tomatoes. Such tomato varieties share a high level of plastid biogenesis and their ripe-red tomatoes are characterized by a substantially high content of antioxidants, such as carotenoids and flavonoids (Yen et al. 1997). These tomatoes also harbor a significantly higher concentration of water-soluble vitamins, such as vitamin C (Mochizuki and Kamimura 1984). The *dg* mutation is considered to be superior over most *hp* mutations because it usually produces tomatoes with higher carotenoid levels, and plants that are more acceptable in several horticultural terms. Numerous studies have examined the high carotenoid content and genetics of the various *hp* and *dg* mutants (Mochizuki and Kamimura 1984; Wann et al. 1985; Yen et al. 1997; Mustilli et al. 1999). However, the molecular regulation of carotenogenesis in such mutant tomatoes still remains obscure.

Historically, the nomenclature of the *hp* and *dg* mutants has been somewhat confusing as was recently summarized (Jones et al. 2001). Also, for years it has been speculated that *hp* and *dg* represent mutations in structural genes of the carotenoid biosynthesis pathway (Stevens and Rick 1986). Although the photomorphogenic nature of these mutants was elucidated several years ago (Mochizuki and Kamimura 1984; Peters et al. 1989, 1992), its possible link to carotenogenesis was largely ignored. A study, published recently, initiated a conceptual link between carotenogenesis control and photomorphogenesis by demonstrating that the phenotype of the tomato *hp-2* and *hp-2*^{j} mutants is caused by mutations in the tomato homolog of *DEETIOLATED1* (Mustilli et al. 1999). The similarities in the pleiotropic phenotypes between the *dg* mutation and several of the known *hp* mutations were recognized by Mochizuki and Kamimura (1984), but the molecular link was missing prior to our research.

In a comprehensive allele-test, carried out in this research, we demonstrate that the *dg* mutation is allelic to *hp-2*^{j}. Furthermore, in a linkage analysis we confirmed that the characteristic photomorphogenic phenotype of *dg* mutants is highly associated with the mutation observed at the *DET1* locus of *dg* genotypes. These results therefore inferred that the gene encoding the *dg* phenotype is located on tomato chromosome 1, and suggested that *dg* is a mutation of the tomato *DET1* gene. Based on this assumption we analyzed the sequence of the *DET1* gene in three *dg* mutant accessions, including an original *dg* mutant plant in the cultivar Manapal background. These sequence analyses revealed that a single Adenine-to-Thymine transversion has occurred in the second exon of the *DET1* gene in all of these *dg* mutants. This single-base transversion leads to the substitution of Aspargine^{34}-to-Isoleucine^{34}. Alignment of the deduced amino-acid sequences of *L. esculentum* DET1, *A. thaliana* DET1 and the mammalian sequence tags (ESTs) with homology to the DET1 protein, indicated that Aspargine^{34} is highly conserved among these species (Mustilli et al. 1999). Furthermore, Aspargine^{34} remained conserved in the multiple alignment of the deduced amino-acid sequence of *Oryza sativa* DET1 (GenBank accession BAB16336) to the above sequences (data not presented), indicating that Aspargine^{34} may be important for the biological action of the DET1 protein.

Interestingly, the mutation, discovered in this study, is the first to be located at the N-terminus of the tomato DET1 protein. This is in contrast to the *hp-2* and *hp-2*^{j} mutations, which are located at the C-terminus of the protein, suggesting that, in tomato as in *Arabidopsis*, both ends of the protein are important for its function(s). In fact, all known *det1* mutations in *Arabidopsis* have been mapped to the ends of the molecule (Pepper et al. 1994). The *Arabidopsis det1-5* mutation is located in the same C-terminus tail of the protein as the tomato *hp-2*^{j}, while *det1-4* in *Arabidopsis* and *dg* in tomato are located in the exon 2 of the gene, corresponding to the N-terminal domain of the protein. In spite of the similarities between the tomato *dg*, *hp-2* and *hp-2*^{j}, and the *Arabidopsis det1* mutations, both in their pleiotropic phenotypic display and in their molecular locations along the protein, they differ in their light requirements. While the *Arabidopsis* mutations display their phenotype under total darkness, the tomato mutations require a limited illumination at the phytochrome functional spectrum (Red/Far Red), in order to display their phenotypic expression (Peters et al. 1992). Indeed, in a full accordance with the light-requirement specificity, the tomato mutations *hp-2* and *hp-2*^{j} do need active phytochromes for a functional display, while the *Arabidopsis* mutations do not (Pepper et al.1994; Mustilli et al. 1999).

The apparent effects of mutations at the tomato *DET1* locus on the increased production of health-promoting carotenoids, flavonoids and vitamins has been thoroughly documented (Mochizuki and Kamimura 1984; Wann et al. 1985; Yen et al. 1997). Such effects suggest that genes active at the light signal-transduction cascade, may be important candidate-genes to associate with quantitative trait loci affecting such important metabolite levels in the tomato fruit. *DET1* homologs were also found in species distantly related to the tomato, such as the human and mouse. This suggests that effects of light-responsive genes on the production of health-promoting compounds should not be ignored in other plant species as well. From such a practical point of view, this study provides a polymorphic DNA marker that can be used as a marker-assisted selection tool for the identification of the *dg* mutant allele. Such a tool can aid the introgression of the *dg* mutation into various genetic backgrounds for the purpose of improving fruit quality and the nutritional value of tomatoes.

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