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ORIGINAL PAPER

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The tomato homolog of the gene encoding UV-damaged DNA binding protein 1 (DDB1) underlined as the gene that causes the *high pigment-1* mutant phenotype

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Abstract A tomato EST sequence, highly homologous to the human and *Arabidopsis thaliana* UV-damaged DNA binding protein 1 (DDB1), was mapped to the centromeric region of the tomato chromosome 2. This region was previously shown to harbor the *HP-1* gene, encoding the *high pigment-1* (*hp-1*) and the *high pigment-1^w* (*hp-1^w*) mutant phenotypes. Recent results also show that the *A. thaliana* DDB1 protein interacts both genetically and biochemically with the protein encoded by *DEETIOLATED1*, a gene carrying three tomato mutations that are in many respects isophenotypic to *hp-1*: *high pigment-2* (*hp-2*), *high pigment-2^j* (*hp-2^j*) and *dark green* (*dg*). The entire coding region of the *DDB1* gene was sequenced in an *hp-1* mutant and its near-isogenic normal plant in the cv. Ailsa Craig background, and also in an *hp-1^w* mutant and its isogenic normal plant in the GT breeding line background. Sequence analysis revealed a single A^{931}-to-T^{931} base transversion in the coding sequence of the *DDB1* gene in the *hp-1* mutant plants. This transversion results in the substitution of the conserved asparagine at position 311 to a tyrosine residue. In the *hp-1^w* mutant, on the other hand, a single G^{2392}-to-A^{2392} transition was observed, resulting in the substitution of the conserved glutamic acid at position 798 to a lysine residue. The single nucleotide polymorphism that differentiates *hp-1* mutant and normal plants in the cv. Ailsa Craig background was used to design a pyrosequencing genotyping system. Analysis of a resource F_2 population segregating for the *hp-1* mutation revealed a very strong linkage association between the *DDB1* locus and the photomorphogenic response of the seedlings, measured as hypocotyl length (25<LOD score<26, R^2=62.8%). These results strongly support the hypothesis that *DDB1* is the gene encoding the *hp-1* and *hp-1^w* mutant phenotypes.

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Introduction

Several photomorphogenic mutants have been described in tomato (*Lycopersicon esculentum*). Among these, plants carrying the monogenic recessive *high pigment* (*hp-1*, *hp-1^w*, *hp-2*, and *hp-2^j*) and *dark green* (*dg*) mutations are characterized by their exaggerated light responsiveness. These mutants display higher anthocyanin levels, shorter hypocotyls, and higher fruit pigmentation than their semi-isogenic wild-type counterparts (Mochizuki and Kamimura 1984; Wann et al. 1985; Peters et al. 1989; Mustilli et al. 1999; Levin et al. 2003). The high pigmentation of the fruits of these mutants is due to significantly elevated levels of carotenoids, primarily lycopene, and of several flavonoids in the mature ripe red fruit. Because of their effect on fruit color, attributed to enhanced 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; http://www.lycored.com/).

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-1^w* mutant appeared among the progeny of a plant raised from ethyl methanesulfonate (EMS)-treated seeds of the genotype GT (Peters et al. 1989), the *hp-2* mutant was reported in the Italian San Marzano variety in 1975 (Soressi 1975), the *hp-2^j* mutant was found among progeny of a T-DNA-transformed plant (cv. Moneymaker) (van Tuinen et al. 1997), and the *dg* mutant appeared in trellised plantings of the Manapal variety (Konsler 1973). Despite some initial confusion, it is now clear that there are two *HP* genes *HP-1* and *HP*-2 in the tomato genome, that map to chromosomes 2 and 1, respectively (van Tuinen et al. 1997; Yen et al. 1997). At each of these loci, two of the above mentioned mutant alleles have been

initially identified: *hp-1* and *hp-1^w*, *hp-2* and *hp-2^j* (Kerckhoffs 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 a 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 shown 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 nuclear localization sequence, and it presumably results in mislocalization of the protein. The milder phenotypic display of 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).

A comprehensive allele test, carried out by our group, has recently shown that the tomato mutations *dg* and *hp-2^j* are allelic, which is consistent with the exaggerated photomorphogenic de-etiolation response of *dg* mutants grown under modulated light conditions. Sequence analysis of the *DET1* gene in *dg* mutants revealed a single A-to-T base transversion in the second exon of the *DET1* gene relative to the normal wild-type sequence. This transversion results in a substitution of the conserved asparagine at position 34 by isoleucine and strongly supports the hypothesis that the tomato *dg* mutation is a novel allele of the tomato homolog of *DET1* gene and, therefore, of the *HP-2* locus (Levin et al. 2003).

The gene encoding the *hp-1* and *hp-1^w* mutant phenotypes has not been identified prior to the present study, although its map location has been clearly characterized (Yen et al. 1997). Our findings show that *hp-1* and *hp-1*^{w} are alternative alleles at the tomato homolog of the *Arabidopsis thaliana* gene encoding UV-damaged DNA binding protein 1 (DDB1), recently shown to interact both biochemically and genetically with the DET1 protein (Schroeder et al. 2002). Our present findings are based on *DDB1* mapping results, detailed sequence analyses of the tomato *DDB1* gene cloned from *hp-1* and *hp-1^w* mutants and their corresponding isogenic normal plants, and on a linkage analysis based on the single nucleotide DNA polymorphism (SNP) obtained between *hp-1* mutants and their near-isogenic wild-type plants in the cv. Ailsa Craig background. In addition, the present study provided the basis for the generation of polymorphic, PCR-based DNA markers that could be used for marker-assisted selection of *hp-1* and *hp-1^w* mutant plants at the seedling stage. This was demonstrated here by developing a co-dominant DNA marker that distinguished between the *hp-1* and normal alleles by means of the pyrosequencing genotyping methodology.

Materials and methods

Plant material and crosses

Seeds from the normal open pollinated tomato (*Lycopersicon esculentum*) cv. Ailsa Craig and a line near-isogenic and homozygous for the *hp-1* mutation were kindly provided by J.J. Giovannoni, of the Boyce Thompson Institute for Plant Research, Ithaca, N.Y..

Seeds from cv. Rutgers homozygous for the *hp-1* mutation (LA3004), as well as seeds from *hp-1^w/hp-1^w* mutant plants and their isogenic normal plants in the GT background (LA4012 and LA4011, respectively), were provided by R.T. Chetelat of the Tomato Genetics Cooperative, University of California at Davis, Calif.. The genotype GT is a tomato breeding line, resistant to mosaic virus, and similar in morphology to cv. Moneymaker, originally obtained from Deruiterzonen, Bleiswijk, The Netherlands (Koornneef et al. 1990). The *hp-1^w/hp-1^w* mutant plants appeared among progeny of a plant raised from EMS-treated seeds of the genotype GT (Peters et al. 1989). Therefore, these plants are highly isogenic to the normal GT genotype. Mutant *hp-1^w/hp-1^w* plants show a more extreme phenotype than *hp-1/hp-1* plants, and it has also been clearly shown that *hp-1* and *hp-1^w* are allelic (Peters et al. 1989).

A processing *hp-1/hp-1* mutant hybrid, LRT89, two *hp-1/hp-1* breeding lines, L525 and L527, and a normal breeding line, N671, were developed by the late R. Frankel, D. Lapushner and I. Levin at the Volcani Center. Seeds from two *hp-1/hp-1* processing hybrids, HA3501 and HA3502, developed by Hazera Genetics, Israel, were provided by Mr. Ezri Peleg. Seeds of the heterozygous *hp-1/+* cultivar, cv. 124, were also provided by Hazera Genetics. Several normal +/+ tomato cultivars used in the present study; Moneymaker, M82, Brigade, VF-36, 189, Manapal, NC8288 and Florida, were grown from seed stocks available at the Volcani Center. DNA was also extracted from single plants of AB427, AB510 and AB747, three *hp-1/hp-1* processing hybrids developed by AB Seeds, Israel.

Normal cv. Ailsa Craig plants were crossed with their near-isogenic *hp-1* mutant plants to yield F_1 seeds. The resulting F_1 plants were allowed to self-pollinate to yield F_2 seeds. A sample of 123 F_2 seedlings was used for the linkage analysis carried out in this study.

Genomic DNA extraction and Southern blot hybridization

Genomic DNA was extracted from individual plants according to Fulton et al. (1995). To determine the copy number of the *DDB1* gene in the tomato genome, Southern blot hybridization was carried out according to the following procedure: genomic DNA extracted from both *L. esculentum* (cv. M82) and *L. pennellii* (LA716) was digested with *Eco*RI, *Eco*RV, *Dra*I, *Hae*III, *Sca*I, and *Mva*I restriction endonucleases. Following electrophoresis in 1.0% agarose gels and transfer onto nylon membranes, the DNA was hybridized with a ^{32}P-labeled DNA probe containing 1,346bp of the 5$'$ coding sequence of the *DDB1* gene. Probe labeling was carried out using Prime-It RmT kits (Stratagene, La Jolla, Calif.) and the hybridization was carried out overnight at 65°C in hybridization buffer (7% SDS, 1% BSA, 1mM EDTA, 0.25M $NaP0_4$, pH 7.2). Hybridized membranes were washed once at low stringency (2×SSC, 0.1% SDS), once at moderate stringency (1×SSC, 0.1% SDS) and once at semi-high stringency (0.5×SSC, 0.1% SDS) for 20min each wash, and exposed on PhosphorImager storage screens (Molecular Dynamics Sunnyvale, Calif.).

Design of PCR primers

Sequence analysis and locus-specific primer design were carried out with the DNAMAN sequence analysis software v4.1 (Lynnon BioSoft, Quebec). All DNA primers used during the course of this study were purchased from the M.B.C. Molecular Biology Center, Ness-Ziona, Israel.

PCR reactions

PCR reactions were used for mapping, cloning and amplification of DNA products for direct sequencing and pyrosequencing. For all of these purposes, the amplification reactions (in a 25 μl final volume) were performed with 10ng template DNA, 25mM TAPS (pH 9.3 at 25°C), 50mM KCl, 2mM $MgCl_2$, 1mM β-mercaptoethanol, 0.2mM of each of the four deoxyribonucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 10 pmol of each of two primers and 1 U of thermostable *Taq* DNA polymerase (SuperNova *Taq* polymerase, Madi, Rishon LeZion, Israel). Reactions were carried out in an automated thermocycler (MJ Research, Watertown, Mass.).

For mapping and direct sequencing, initial incubation was at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 58°C for 30s, and elongation at 72°C for 1 to 2min, depending upon the PCR product size. A final elongation step at 72°C was carried out for 5min after completion of the above cycles. The PCR amplification products were visualized by electrophoresis in 1.0% agarose gels and detected by staining with ethidium bromide.

For the PCR amplification preceding the pyrosequencing reaction, initial incubation was at 94°C for 2min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 57°C for 30s, and elongation at 72°C for 20s. A final elongation step at 72°C was carried out for 5min after completion of the above cycles.

Mapping the *DDB1* gene

DDB1 was mapped by means of *Lycopersicon pennellii* introgression lines (Eshed et al. 1992). The mapping procedure was described in detail by Levin et al. (2000).

Cloning and sequencing of the tomato *DDB1* cDNA from *hp-1* and *hp-1*^{w} mutant plants

Total RNA was extracted from 25mg of leaf tissue of individual *hp-1* and *hp-1*^{w} mutant seedlings and their near-isogenic open pollinated wild-type genotypes (Ailsa Craig and GT, respectively). The RNA extraction was carried out with the TRIzol reagent system (GibcoBRL Life Technologies, Gaithersburg, Md.). Total RNA was used as the template for first-strand cDNA synthesis using the Superscript pre-amplification system (GibcoBRL Life Technologies). This cDNA was used as a template in PCR reactions to amplify overlapping fragments of the gene encoding the tomato DDB1 from both mutant and normal genetic accessions. The PCR products were then sequenced, either directly or after cloning into pGEM-T Easy Vector by means of the pGEM-T Easy Vector System, according to the manufacturer's recommendations (Promega Corporation, Madison, Wis.). After cloning into pGEM-T Easy, four or five independent clones of each of the overlapping amplified fragments were sequenced, using the vector T7 and SP6 primer sites, and primers complementary to the tomato *DDB1* gene. Whenever direct sequencing was used, at least two PCR products representing each primer combination, complementary to the tomato *DDB1* gene, were sequenced. Sequencing was carried out with an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, Calif.).

The 3′ region of the tomato *DDB1* gene was directly sequenced by using overlapping fragments amplified with primers complementary to the Institute of Genomic Research (TIGR) data base accession TC117372 (http://www.tigr.org/) that is highly homologous to both copies of the *A. thaliana DDB1* gene. These primers are presented in Table 1.

The 5′ region of the *DDB1* gene was initially cloned from a pBluescript SK(+/-) phagemid cDNA library with the following primers: T7=5′-GTAATACGACTCACTATAGGGC-3′ and 5TDDB_R= 5′-CTGGACTTGAGAATTGAAGCCT-3′.

This cDNA library, kindly provided by R. Barg and Y. Salts of the Volcani Center, was prepared from young parthenocarpic fruits of 4 to 6mm in diameter (around 4 to 8 days post-anthesis) derived from the facultative parthenocarpic determinate line L-179 (*pat-2/pat-2*). This line was described previously in Barg et al. (1990).

Table 1 Forward (F) and reverse (R) primers, complementary to TIGR database accession TC117372, used to sequence the 3′ region of the tomato *DDB1* gene

Primer code	Primer sequence
5TDDB F	5′-ACGACCTATCGTGGACTTCTGT-3′
5TDDB R	5′-CTGGACTTGAGAATTGAAGCCT-3′
In5TDDB F	5′-GAGCCTATAAGGATGGATCAC-3′
ATDDB F	5′-CAGCAGTTGGAATGTGGACAG-3′
MTDDB F	5′-GCAATCGCTAAAGAAGGTGAGT-3′
MTDDB R	5′-GCATTATAGTCTCTGGCTCGCT-3′
InmTDDB F	5′-GGACATTTGCTCTATGCAGT-3′
InmTDDB R	5′-AGGCATTTAGAGAGTAGACAGC-3′
TDDB F	5′-TTTGGAGAAGCTGCAGACAA-3′
TDDB R	5′-CACAACCTCACAGAAGAAGAAG-3′
In3TDDB R	5′-CCACTCTCTTCATTAGTTCCTC-3′

The 5′ region of the tomato *DDB1* gene from *hp1/hp1* and *hp1*^{w}/*hp1*^{w} mutants and their corresponding near-isogenic normal lines was directly sequenced, initially with the above primer (5TDDB_R) and the primer TDB_UTR=5′-ATAGCGGGAAGAGGGAAGATAC-3′, complementary to the 5′ UTR of the tomato *DDB1* gene. Several overlapping primers complementary to the above fragment, such as those used for pyrosequencing genotyping (see below), were used for sequence verification of the 5′ coding sequence of the tomato *DDB1* gene.

Linkage analysis

A linkage analysis study between the tomato *DDB1* locus and the exaggerated photomorphogenic de-etiolation response characterizing *hp-1* mutant was carried out using F_2 seeds of a cross between the *hp-1* mutant plants and wild-type plants (cv. Ailsa Craig). These seeds were allowed to germinate under a yellow plastic screen that prevented transmission of light of wavelengths under 500nm (Mochizuki and Kamimura 1984), in an environmentally controlled growth chamber (25°C day/18°C night). These germination and initial growth conditions result in an exaggeration of the hypocotyl length differences between the mutant and normal plants (Mochizuki and Kamimura 1984). The hypocotyl lengths of individual F_2 seedlings were measured 8 days after sowing, and their genotype determined with the pyrosequencing-based DNA marker developed in this study.

Pyrosequencing genotyping

A pyrosequencing genotyping system, extensively reviewed by Ronaghi (2001), was developed in the present study, based on the SNP we discovered between the *hp1/hp1* mutant and its near-isogenic normal lines in the cv. Ailsa Craig background. For this purpose a genomic fragment containing the SNP was cloned and sequenced. The sequence of this genomic fragment is presented in Fig. 1. The biotin-labeled forward primer for this reaction was 5′-TGTTTTCCAGAGTTACCGGACT-3′; the reverse primer was 5′-TAGCTTGAGCCAATGAAGACAA-3′, and the sequencing primer was 5′-ATGAAGACAAAAGCAT-3′. The amplicon size in this reaction was 106bp.

The PCR amplification reaction preceding the pyrosequencing reaction was as described above (see PCR reactions). Two picomoles of the sequencing primer was added to the amplification reaction prior to the pyrosequencing analysis which was carried out with a MegaBASE 1000 instrument (Danyel Biotech, Nes Ziona, Israel). Because the sequencing primer is in the reverse orientation, the normal genotype is characterized by T whereas the homozygous mutant *hp-1* genotype is characterized by A at the SNP location, as shown in Fig. 2.

CTCATGAGAAGGAGAAGTGCGTCAGCATTTTCTAGACTGTCATTTCTACTTTAGCTGAGT
TGCTGGGAATGAAATCTTCTCTTGTACCCCTGCCTGGTTGCTGGAATAAAAATGTTTAAT
TTGGATTGTTAACCTGTTTTCCAGAGTTACCGGACTCAAAATTGAGCTACTGGGGGAAAC
TTCTATTGCATCAACCATATCATACCTAGAC**A/T***ATGCTTTTGTCTTCATT*GGCTCAAG
CTACGGAGATTCACAGGTACTTTTAACTGTTGAGTGCATCTTGGTGCAATAAGTTGGTTT
TTAGAGCTGCCTTATTGTATTTTCCATACAGTAGCCTTTCATTCATTTGGAACATTGAGG
TTTTAAATTTCAGTTGCCTATTTCTGGTGGTGCTTCATATTTCACAGTTCCACTAATATT
TTTGAATTCACGTTTAGCTTGTAAAGCTCAATCTCCAGCCTGACACCAAA

Fig. 1 The genomic fragment used to design pyrosequencing primers for the *hp-1* mutation (the single nucleotide polymorphism is in *underlined large bold letters*, the forward and the reverse primers are *underlined* and the sequencing primer is in *italic*)

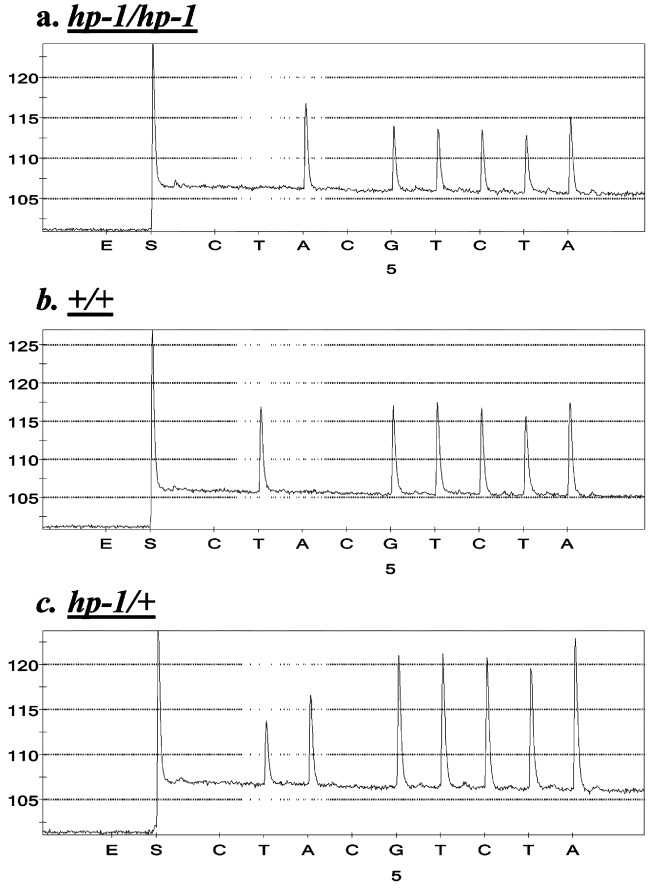

Fig. 2 Typical pyrosequencing genotyping results for the *hp-1* mutation at the *DDB1* locus. (Note that because of the reverse orientation of the sequencing primer, the mutant genotype is characterized by A and the normal genotype by T)

Statistical analyses

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

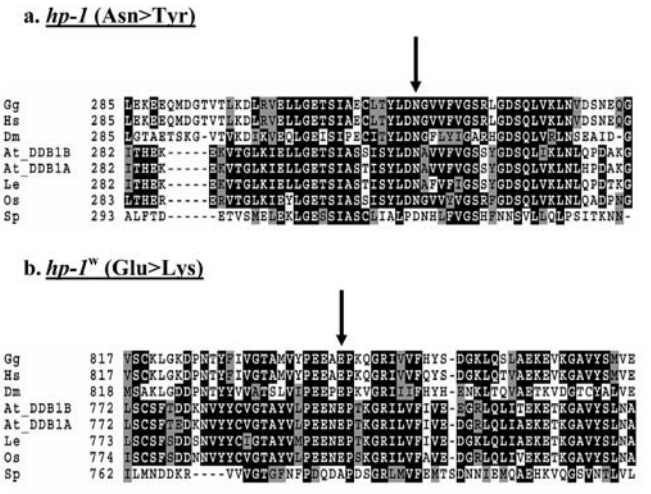

Fig. 3a, b Partial ClustalW protein alignment of DDB1 showing the location of the *hp-1* (**a**) and *hp-1*^{w} (**b**) amino acid substitutions. Presented are *Arabidopsis* DDB1A (At_DDB1A=NP_192451), *Arabidopsis* DDB1B (At_DDB1B=NP_193842), tomato cv. Ailsa Craig and GT line (Le=AAR20885), rice (Os=BAB20761), human (Hs=DDB1_Human), *Drosophila* (Dm=XP_081186), chicken (Gg=BAC56999), and *Schizosaccharomyces pombe* (Sp=NP_593580). Identical residues are *shaded black* whereas similar residues are *shaded gray*

Results

Identification and cloning of the tomato homolog of *DDB1*

The full-length coding region of the tomato *DDB1* gene, Fig. 3 and GenBank accessions AAR20885 and AY452480, was identified, cloned and sequence characterized according to the following considerations. The DDB protein is a heterodimer consisting of two subunits, DDB1 and DDB2. Unlike those of rice, chicken, humans, mouse, *Drosophila* and *Schizosaccharomyces pombe*, the *A. thaliana* genome harbors two copies of the *DDB1* gene that share 89% and 87% identities at the amino acid and nucleotide level, respectively (Schroeder et al. 2002; Zolezzi et al. 2002; Fu et al. 2003; Ishibashi et al. 2003): *DDB1A*, and *DDB1B*, translated into 1,088 amino acid proteins (GenBank protein accessions NP_192451 and NP_193842, respectively). When each of these two protein accessions was used as a query in tblastn analysis against the TIGR database (http://www.tigr.org/) containing tomato expressed sequence tags (EST), both revealed two highly homologous sequences: TC117371 (394bp) and TC117372 (2,206bp). The *A. thaliana* accession NP_192451 was found to share 87% and 86% identities with the tomato TC117371 and TC117372 accessions, respectively. Accession NP_193842, on the other hand, shared 87% and 83% identities with the tomato TC117371 and TC117372 accessions, respectively. Careful sequence analysis, based initially on the longer TIGR accession, TC117372, and later on the single gene that we had cloned from a cDNA library, made it clear to us that

the two tomato TIGR accessions, TC117371 and TC117372, were complementary to the same gene sequence. Moreover, Southern blot transfer and hybridization of tomato genomic DNA, with the *DDB1* gene sequence as a probe, revealed that it is unlikely that additional copies of *DDB1* gene exist in the tomato genome (data not presented).

Mapping of the tomato *DDB1*

DDB1 was mapped by means of *Lycopersicon pennellii* introgression lines (Eshed et al. 1992). DNA extracted from individual plants of each of the introgression lines, including their original parental lines M82 and *L. pennellii*, were used as templates in PCR reactions. The primers used for these reactions were MTDDB F and MTDDB R (Table 1). These primers were derived from the TIGR EST database accession TC117372 (http://www.tigr.org/) that was found to be highly homologous to both copies of the *A. thaliana DDB1* gene. The PCR reaction generated a single monomorphic band. To obtain polymorphisms between M82 and *L. pennellii*, which could be also traced among individual introgression lines, PCR products were digested by several endonucleases. Our results demonstrate that digestion with *Pst*I endonuclease generated the required polymorphism (Fig. 4).

Partial mapping results that include the approximate map location of the tomato *DDB1* gene are presented in Fig. 4. These results indicate that the *DDB1* is located on the tomato chromosome 2 in the introgression line that harbors the *HP-1* gene (Yen et al. 1997).

Sequence characterization of the tomato *DDB1* in *hp-1* and *hp-1*^{w} mutants

We used several forward and reverse primers (Table 1), complementary to the 3′ region of the tomato *DDB1* gene (TIGR accession TC117372), to perform direct sequencing on cDNA prepared from leaves of *hp-1/ hp-1* and normal seedlings in the Ailsa Craig background. No polymorphism was obtained between mutant *hp-1* and normal plants in this region. We therefore cloned and thoroughly sequenced the 5′ region of the *DDB1* gene in the two genotypes as well. Computerized translation of all the sequence results showed that the tomato DDB1 is a 1,090 amino acid protein (GenBank accessions AAR20885 and AY452480). Sequence analysis of the *DDB1* coding sequence from *hp-1* and its near-isogenic normal genotype revealed a single A^{931}-to-T^{931} base transversion in the coding sequence of the *DDB1* gene of the mutant *hp-1* plants. This transversion resulted in a substitution of the conserved asparagine at position 311 by a tyrosine residue (Fig. 3).

Based on the sequence information obtained in the Ailsa Craig background, we have also sequenced the entire coding region of the *DDB1* gene in the *hp-1*^{w} mutant and its isogenic normal counterpart in the GT background. Because *hp-1*^{w} is allelic to *hp-1*, a major mutation in the coding sequence of the *DDB1* gene in *hp-1*^{w} mutants would strongly support the hypothesis that the tomato *DDB1* gene causes both the *hp-1* and *hp-1*^{w} mutant phenotypes. Indeed, a single $G^{2,392}$-to-$A^{2,392}$ transition was observed in the *DDB1* coding sequence of the *hp-1*^{w} mutant plants, resulting in the substitution of the conserved glutamic acid at position 798 by a lysine residue (Fig. 3).

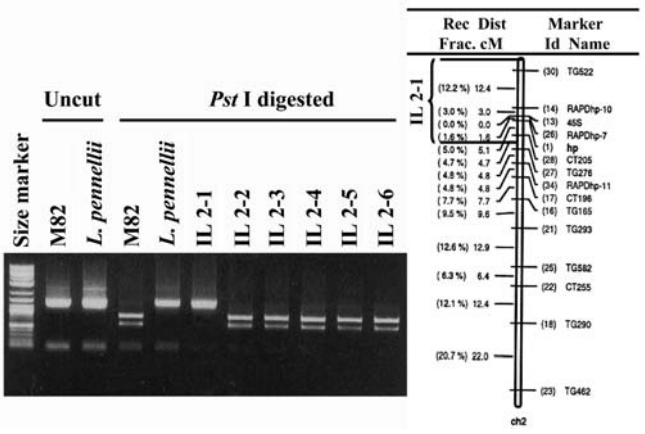

Fig. 4 Partial mapping results of the tomato *DDB1* gene [map of the tomato chromosome 2, showing the location of the *HP-1* gene (*hp*), adopted from Yen et al. (1997)]

Genotyping of lines and cultivars

We genotyped 19 lines or cultivars, obtained from various sources, by a combination of direct sequencing and pyrosequencing methods (Fig. 2). Among them were, to the best of our knowledge, a single heterozygous *hp-1/+*, ten *hp-1/hp-1* and eight normal +/+ accessions. We found complete agreement between the SNP identified in the *DDB1* gene and the known genotypes of the plants at the *HP-1* locus (results not presented).

Linkage analysis between the *DDB1* locus and the photomorphogenic response

A linkage analysis study was carried out to test the association between the *DDB1* locus and the characteristic hypersensitive photomorphogenic response displayed by *hp-1* mutant seedlings (i.e., the inhibition of the hypocotyl elongation phenotype). For this purpose, F_2 seeds of a cross between *hp-1/hp-1* mutant plants and normal plants (cv. Ailsa Craig) were germinated in a controlled growth chamber under a yellow plastic screen. These plastic screens trigger de-etiolation by exclusion of blue light, while allowing exposure to the phytochrome functional spectrum (red/far red), and result in exaggeration of the hypocotyl length differences between the

Table 2 Linkage analysis between the tomato *DDB1* locus and the photomorphogenic response displayed by *hp-1* mutant seedlings

Genotype	*N*	Hypocotyl length (cm, mean±SE)*	LOD score	R^2
+/+	35	9.6^{A}±0.2	25<LOD<26	62.8%
hp-1/+	68	8.7^{B}±0.2	–	–
hp-1/hp-1	20	4.2^{C}±0.2	–	–

* Different superscript letters indicate statistically significant differences among means (P<0.05) according to the Tukey-Kramer HSD test (Kramer 1956)

mutant and normal plants (Mochizuki and Kamimura, 1984; Peters et al. 1992).

Eight days after sowing, the hypocotyl lengths of individual seedlings were recorded, and their *DDB1* locus was genotyped with aid of the pyrosequencing DNA marker, as described above. The results demonstrate a clear association between the *DDB1* locus and hypocotyl length (Table 2). Homozygous recessive *hp-1/hp-1* seedlings displayed a highly significant inhibition of hypocotyl elongation, indicative of a more exaggerated photomorphogenic de-etiolation response, than those of the other two other genotypic groups (25<LOD score<26, R^2=62.8%). These results confirm that the mutation identified in the *DDB1* locus of *hp-1* mutant plants is associated with one of its main characteristic phenotypes, i.e., inhibited hypocotyl elongation in seedlings. Interestingly, a slight partially dominant effect was obtained in this study for the *hp-1* allele. This effect can be noted from the statistical significant differences obtained between the +/+ and *hp-1*/+ group means (Table 2).

Discussion

Plants respond to light by modulating their developmental processes in an array of interactions that are commonly and collectively referred to as photomorphogenesis. Photomorphogenic mutants have proven to be an excellent tool in studying the complex interactions between light and plant development. In general, these mutants may be classified either as defective in photoreceptors, or altered in some elements of the light signal transduction chain (Chory 1993). Photomorphogenesis has been intensively studied in the model species *A. thaliana*. It is however difficult to carry out the appropriate biochemical studies or to test hypotheses related to the interaction between light and fruit quality, on its small, dry fruits. The tomato, on the other hand, is characterized by a fleshy fruit that accumulates phytochemicals. The tomato homolog of the photomorphogenic *DEETIOLATED1* (*DET1*) gene has recently been characterized as the gene carrying three light-sensitive mutations: *high pigment-2* (*hp-2*), *high pigment-2^j* (*hp-2^j*) and, recently, *dark green* (*dg*), but these have been only partially characterized (Mustilli et al. 1999; Levin et al. 2003). The exaggerated light responsiveness that typifies these mutants leads to significantly higher anthocyanin levels in their developing seedlings than in those of their semi-isogenic wild-type counterparts. These mutants are also characterized by higher fruit and foliage pigmentation. The high fruit pigmentation of these mutants is due to significantly elevated levels of carotenoids, primarily lycopene, in the mature ripe-red fruit (Mochizuki and Kamimura 1984; Wann et al. 1985; Yen et al. 1997; Levin et al. 2003).

Interestingly, the *hp* and *dg* mutant plants are also characterized by overproduction of several flavonoids and of vitamin C in their mature fruit (Mochizuki and Kamimura 1984; Yen et al. 1997). This overproduction is associated with increased plastid biogenesis, which suggests that overproduction of other desirable metabolites may be expected in such mutants. These mutations, therefore demonstrate a conceptual link between photomorphogenesis and overproduction of health-promoting metabolites. Tomato plants carrying the *high pigment* (*hp*) and *dark green* (*dg*) mutations therefore represent a unique, non-GMO, genetic platform for the production of functional tomatoes.

Historically, the nomenclature of the *hp* and *dg* mutants has been somewhat confusing, a situation that was recently summarized by 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; Peters et al. 1992), its possible link to carotenogenesis has been largely ignored. An elegant study, published recently, addressed the concept of a link between carotenogenesis control and photomorphogenesis, by demonstrating that the phenotype of the tomato *hp-2* and *hp-2^j* mutants was caused by mutations in the tomato homolog of *DET1*, a gene encoding a negative regulator of photomorphogenesis (Chory 1993; Mustilli et al. 1999). We have recently identified *dg* as an additional mutation at the *DET1* locus (Levin et al. 2003). The similarities in the pleiotropic phenotypes of all known *hp* mutations, as expressed in seedlings, mature plants, and developing and ripe-red fruits, were recognized (Mochizuki and Kamimura 1984), but the molecular link was incomplete prior to our present study. These phenotypic similarities also led us to map candidate photomorphogenic genes, in an attempt to identify the gene that encodes the *hp-1* mutant phenotype (data not shown). However, none of those genes was found to map in close association with the *HP-1* locus. An epitope-tagging approach, recently applied in *A. thaliana*, showed that the DET1 protein is localized to the nucleus and forms an approximately 350 kDa complex, which is required for full DET1 activity (Schroeder et al. 2002). A 120 kDa protein that is the plant homolog of DDB1, a protein

implicated in the human disease *Xeroderma pigmentosum*, was affinity purified from that complex. Both the demonstrated molecular interaction between the DET1 and DDB1 proteins and the observed phenotypic similarities between mutants at the *DET1* gene and mutants at the *HP-1* locus led us to test the hypothesis that the *DDB1* gene encodes the mutant phenotypes at the *HP-1* locus.

The comprehensive molecular and genetic tests we have carried out in this study show that *hp-1* mutant plants are characterized by a substitution of asparagine to tyrosine at residue 311 in the DDB1 protein (Fig. 3). We also show that in the more phenotypically extreme *hp-1^w* mutation, previously shown to be allelic to *hp-1* (Peters et al. 1989), an acidic residue, glutamic acid^{798}, is substituted by a basic residue, lysine, in the same protein (Fig. 3). Both asparagine^{311} and glutamic acid^{798} are highly conserved residues in the DDB1 protein, in both plant and animal species (Fig. 3). Furthermore, in a linkage analysis we show that the characteristic photomorphogenic phenotype of early seedling hypocotyl growth of *hp-1* mutants is strongly associated with the mutation observed at the *DDB1* locus of *hp-1/hp-1* genotypes (Table 2). These results therefore suggest that *hp-1* and *hp-1^w* mutant phenotypes are caused by mutations in the tomato homolog of the *A. thaliana DDB1* gene.

DDB is a heterodimer, comprised of two subunits: DDB1 and DDB2. DDB is an important factor involved in DNA repair and cell cycle regulation and it has high affinity for a variety of DNA lesions including UV photoproducts (Fu et al. 2003; Ishibashi et al. 2003). DDB1 was originally identified as a component of the UV-damaged DNA binding activity that is lacking in *X. pigmentosum* complementation group E patients (Chu and Chang 1988; Keeney et al. 1993). The molecular lesions in these patients, however, were later mapped to DDB1's binding partner, DDB2 (Nichols et al. 1996; Hwang et al. 1998). Both DDB1 and DDB2 were found to interact with either histone acetyltransferase (HAT) proteins or HAT complexes (Brand et al. 2001; Datta et al. 2001; Martinez et al. 2001). Recently it was also shown that tomato DET1 interacts with histones, specifically the non-acetylated tail of H2B, both in vitro and in vivo (Benvenuto et al. 2002). These protein interactions, as well as the involvement of DET1 in the expression of many light-regulated genes have led to the suggestion that DET1 and DDB1 regulate gene expression via an interaction with chromatin (Schroeder et al. 2002).

In *A. thaliana* and tomato, *det1* mutants exhibit many characteristics of light-stressed plants. These characteristics were intensively characterized in *A. thaliana*, following a detailed microarray analysis which showed that the expression of many genes associated with various stress responses, including light stress, are misregulated in *det1* mutant plants (Hu et al. 2002; Schroeder et al. 2002). The interaction of DET1 with DDB1, a protein that protects humans from damaging UV rays, as well as our present findings that the light-sensitive *hp-1* and *hp-1^w* mutations are in fact lesions in the gene that encodes DDB1 protein, further reinforce the hypothesis that the roles of these proteins may have evolved from a common mechanism for managing light stress (Schroeder et al. 2002).

Interestingly, a null mutation in the *A. thaliana DDB1A* results in no obvious phenotype, yet it enhances the phenotype of a weak *det1* allele. In *A. thaliana*, however, there are two highly homologous copies of the *DDB1* that share 89% and 87% identities at the amino acid and nucleotide levels, respectively. Therefore, a mutation in *DDB1A* may be compensated for by the other copy, i.e., *DDB1B*. Southern blot hybridization, carried out in our present study (data not shown), indicated that *DDB1* is a single-copy gene in the tomato genome, as has been found also in rice, chicken, humans, mouse, *Drosophila* and *Schizosaccharomyces pombe* (Schroeder et al. 2002; Zolezzi et al. 2002; Fu et al. 2003; Ishibashi et al. 2003). A mutation in such a single-copy gene may generate a much greater phenotypic effect than that in *A. thaliana*, as can be observed in *hp-1* and *hp-1^w* mutants of tomatoes. Differences were also observed between the tomato and *A. thaliana det1* mutants in spite of the 69% DET1 protein identity that exists between them. Whereas the *Arabidopsis* mutations display their phenotype under total darkness, the tomato mutations require an exposure to the phytochrome functional spectrum (red/far red), in order to reveal their phenotype (Peters et al. 1992). Indeed, in full accordance with the light requirement specificity, the tomato *hp* mutations need active phytochromes for functional display whereas the *Arabidopsis* mutations do not (Pepper et al.1994; Mustilli et al. 1999).

The apparent effects of mutations at the tomato *DET1* locus, and in light of the findings of the present study, on the DET1 protein complex, including the DDB1 protein, on the increased production of health-promoting carotenoids, flavonoids and vitamins have already been documented (Mochizuki and Kamimura 1984; Wann et al. 1985; Yen et al. 1997; Levin et al. 2003). Such effects suggest that genes active in the light signal transduction cascade may be important candidate genes for association with quantitative trait loci that affect such important metabolite levels in the tomato fruit. *DET1* and *DDB1* homologs were also found in a wide range of species distantly related to the tomato (Schroeder et al. 2002; Fig. 3). This suggests that effects of light-responsive genes on the production of health-promoting compounds in other plant species should not be ignored. From such a practical point of view, the present study has identified DNA polymorphisms that can be further used as a marker-assisted selection tools for the identification of the *hp-1* and *hp-1^w* mutant alleles and for combining these alleles with alleles that have already been molecularly characterized and mapped to the unlinked *HP-2* gene (Mustilli et al. 1999; Levin et al. 2003). Such tools could aid the introgression of the *hp-1* or *hp-1^w* mutations into various genetic backgrounds for the purpose of improving the fruit quality and nutritional value of tomatoes.

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