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Regulation of Carotenoid Biosynthesis during Tomato Development

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Phytoene synthase (Psy) and phytoene desaturase (Pds) are the first dedicated enzymes of the plant carotenoid biosynthesis pathway. We report here the organ-specific and temporal expression of *PDS* and *PSY* in tomato plants. Light increases the carotenoid content of seedlings but has little effect on *PDS* and *PSY* expression. Expression of both genes is induced in seedlings of the phytoene-accumulating mutant *ghost* and in wild-type seedlings treated with the Pds inhibitor norflurazon. Roots, which contain the lowest levels of carotenoids in the plant, have also the lowest levels of *PDS* and *PSY* expression. In flowers, expression of both genes and carotenoid content are higher in petals and anthers than in sepals and carpels. During flower development, expression of both *PDS* and *PSY* increases more than 10-fold immediately before anthesis. During fruit development, *PSY* expression increases more than 20-fold, but *PDS* expression increases less than threefold. We concluded that *PSY* and *PDS* are differentially regulated by stress and developmental mechanisms that control carotenoid biosynthesis in leaves, flowers, and fruits. We also report that *PDS* maps to chromosome 3, and thus it does not correspond to the *GHOST* locus, which maps to chromosome 11.

INTRODUCTION

Carotenoids are yellow, orange, and red pigments present in all plant tissues. Phytoene, the first carotenoid in the pathway, is synthesized from two molecules of geranylgeranyl diphosphate by the enzyme phytoene synthase (Psy). In the next step of the plant pathway, the enzyme phytoene desaturase (Pds) catalyzes the conversion of phytoene into ζ-carotene. Phytoene is a colorless compound, ζ-carotene is pale yellow, and later compounds are red (lycopene), orange (β-carotene), or yellow (xanthophylls or hydroxylated carotenoids). A byproduct of the pathway is the phytohormone abscisic acid, which modulates plant responses to various developmental and stress signals (Bartley et al., 1991a).

During plant development, carotenoids play dual roles as essential photoprotectants in green tissues and as dispensable colorants in flowers and fruits. When the photosystem is saturated, chlorophyll molecules in the photosynthetic antenna system can react with oxygen to form oxygen singlets, which trigger lethal oxidative reactions. Carotenoids prevent the formation of oxygen singlets by quenching the excited chlorophylls (Krinsky, 1979).

Carotenoids are synthesized and accumulated in plastids, where they bind specific hydrophobic proteins. Although all plastids contain carotenoids, the highest levels of these pigments are found in chloroplasts and chromoplasts. In chloroplasts, carotenoids constitute ~3% of the weight of the thylakoid lipids (Hoober, 1984). Chromoplasts are plastids specialized to accumulate high levels of carotenoids and are responsible for the bright yellow and red colors of many fruits and flowers.

In flowers and fruits, carotenoids are likely to aid in the attraction of pollinating insects and animals that can contribute to seed dispersal. Breeding has been used to increase the carotenoid content of many flowers and fruits. However, fruits of some wild relatives of tomato are not red and many flowers do not accumulate significant amounts of carotenoids in their petals, indicating that these pigments are not essential for the development of flowers and fruits.

Plant genes for enzymes of the carotenoid biosynthesis pathway are nuclear, and the protein products are imported into organelles (reviewed in Bartley et al., 1991a). Molecular probes for carotenoid biosynthesis genes are now becoming available. In the last year, *PDS* has been cloned from soybean (Bartley et al., 1991b), pepper (Hugueney et al., 1992), and tomato (G. E. Bartley and P. A. Scolnik, unpublished data, GenBank accession number M88683; Pecker et al., 1992), and *PSY* was identified as corresponding to pTOM5, a cDNA for a ripening-induced tomato gene (Bartley et al., 1992; Bramley et al., 1992).

Previous reports on carotenoid biosynthesis dealt with the control of *PDS* and *PSY* expression during tomato fruit ripening (Maunders et al., 1987; Pecker et al., 1992). However, the

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low level of expression of these genes precluded studies in other organs. As part of a long-term study of carotenoid biosynthesis in plants, we have now used a sensitive mRNA detection method to study the expression of *PSY* and *PDS* during the development of tomato plants.

RESULTS

Development of an Expression Assay

The reverse transcriptase–polymerase chain reaction (RT-PCR) amplification assay provides the level of sensitivity required to study rare transcripts (Chelly et al., 1988; Kawasaki, 1991; Rappolee et al., 1991).

To study *PSY* and *PDS* expression by RT-PCR, we used reverse transcriptase and RNA isolated from different tomato organs to produce first-strand cDNA. We then amplified the *PDS* and *PSY* cDNAs using 28 cycles of amplification with *Taq* polymerase, and we measured the products by ethidium bromide–induced fluorescence. We designed the primers to flank intron regions. As predicted from the genomic DNA sequences of *PSY* (Ray et al., 1992) and *PDS* (B. Aracri, G. E. Bartley, P. A. Scolnik, and G. Giuliano, manuscript in preparation), amplifications of 100 ng of genomic DNA resulted in bands greater than 1 kb that, as shown in Figure 1, were absent in the amplification of RNA samples. Also, omitting reverse transcriptase abolished the *PDS* and *PSY* bands (data not shown). Thus, the *PDS* and *PSY* bands detected correspond to the amplification of the corresponding mRNAs and not to contamination with genomic DNA. Ray et al. (1992) have reported the sequence of a gene related to pTOM5. Therefore, we designed our primers to be specific for pTOM5 (*PSY*), and restriction digest analyses of the amplified product confirmed the specificity of the reaction (data not shown).

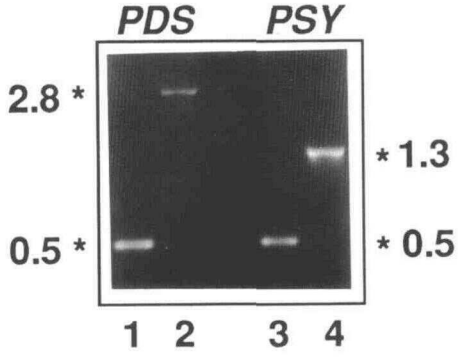

Figure 1. Amplification of *PSY* and *PDS* from Genomic DNA and from cDNA.

PSY and *PDS* were amplified from first-strand cDNA (lanes 1 and 3) and genomic DNA (lanes 2 and 4), as described in Methods. The amplification reactions were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. The amplification products and their size in kilobases are indicated by asterisks.

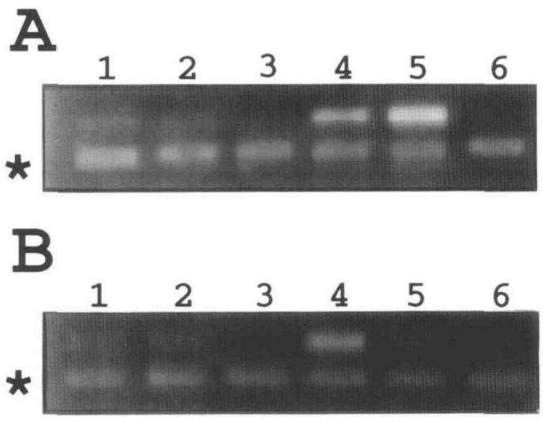

Figure 2. Expression of *PDS* and *PSY* in Mature Plants Studied by RT-PCR.

Lanes 1 contain RT-PCR amplification products from leaf; lanes 2, stem; lanes 3, root; lanes 4, flower; lanes 5, fruit; lanes 6, control with no plant RNA.

(A) Amplification of *PDS*. The upper band (~500 bp) is derived from the *PDS* transcript; the lower band (311 bp, marked by an asterisk) is derived from the artificial control RNA.

(B) Amplification of *PSY*. The upper band corresponds to the *PSY* transcript; the lower band is a control, as given in **(A)**.

As shown in Figure 2, using RT-PCR we were able to detect expression of both genes in all organs of mature tomato plants. The signals from the corresponding genes vary significantly in the different samples. To verify that the efficiency of reverse transcription and amplification was similar in all samples, we included in this experiment an artificial control transcript derived from the Bluescript SK+ plasmid. The intensity of the signal from this control transcript does not vary significantly from sample to sample (Figure 2).

To quantify the response, we constructed a calibration curve with the control transcript and with leaf RNA. The results shown in Figure 3 indicate that the intensity of the RT-PCR product band is proportional to the amount of input RNA, and that leaf RNA does not interfere with the amplification of the artificial control RNA. The response obtained is semilogarithmic instead of linear, as observed with radioactive labeling of RT-PCR products (Singer-Sam et al., 1990). However, we prefer the film-scanning method used here because of its simplicity and high reproducibility.

Control of Expression by Light

Seedlings develop according to a complex process that, in dicotyledonous plants, is triggered by light. Deetiolation of seedlings results in the concurrent formation of leaves, the

development of chloroplasts from undifferentiated plastids, and the 10- to 100-fold induction of expression of a set of nuclear genes. Etiolated seedlings of angiosperms are yellow due to the accumulation of carotenoids, which, unlike the chlorophylls, are synthesized in the dark. Light has a greater than fourfold stimulatory effect on carotenoid accumulation, as shown in Table 1. This value is consistent with observations made in other systems (Kirk, 1978). In contrast, there is a greater than 27-fold increase in chlorophyll upon deetiolation (Table 1).

To test the effect of light on *PSY* and *PDS* expression, we determined the levels of both transcripts in etiolated seedlings exposed to light and in light-grown plants transferred to darkness. The results in Table 2 indicate that *PDS* mRNA levels vary less than twofold during either deetiolation or dark adaptation. *PSY* mRNA levels also remain essentially constant during deetiolation, but they decrease approximately sevenfold upon dark adaptation.

Thus, expression of *PDS* and *PSY* is essentially constitutive during photomorphogenesis. After completion of the photomorphogenesis program, light is only required for expression of *PSY*.

Table 1. Pigment Composition at Various Stages of Development

Sample	Stage[a]	Chlorophyll[b]	Carotenoids[b]
Seedlings	Etiolated	8.0 ± 5	11 ± 1.5
	Light	218.0 ± 13	48 ± 1.5
Petals	2	209.0 ± 20	74 ± 11.0
	3	129.0 ± 15	100 ± 6.0
	4	72.0 ± 5	538 ± 5.0
	5	28.0 ± 5	652 ± 17.0
Fruit pericarp	IG	21.2 ± 2	76 ± 8.0
	MG	8.0 ± 1	25 ± 2.0
	Orange	ND	28 ± 4.0
	Red	ND	284 ± 30.0

[a] Petals and fruits, stages as given in Figure 4; IG, immature green; MG, mature green.

[b] Average of two determinations expressed in micrograms per gram fresh weight of tissue; ND, not determined.

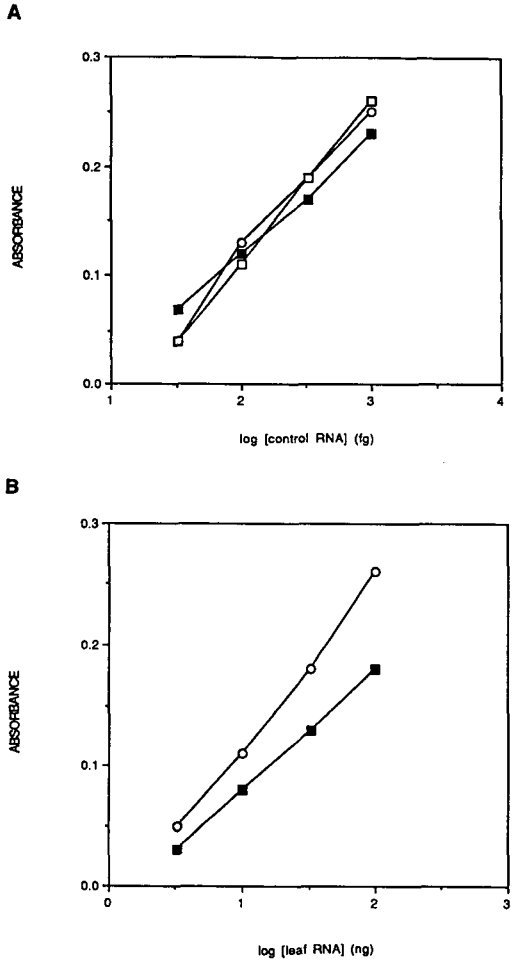

Figure 3. Standard Curves for Quantitation of RNA by RT-PCR.

(A) Amplification by RT-PCR of an external control RNA containing no plant RNA (circles), or 10 ng (open squares) and 100 ng (filled squares) of leaf RNA.

(B) Amounts of RT-PCR products amplified with the indicated quantities of leaf RNA from *PSY* (circles) and *PDS* (squares).

Carotenoid Biosynthesis and Gene Expression

Blocking the biosynthesis of colored carotenoids by chemical or genetic approaches results in lower mRNA levels for several nuclear genes that encode chloroplast proteins (reviewed in Taylor, 1989). We used seedlings of the *ghost* mutant and wild-type seedlings treated with the Pds inhibitor norflurazon to test the effect of blocking carotenoid biosynthesis on *PSY* and *PDS* expression. The *ghost* mutation blocks the desaturation of phytoene (Rick et al., 1959; Scolnik et al., 1987). The results in Table 3 indicate that in seedlings that lack colored carotenoids *PSY* is induced two- to threefold and *PDS* five- to tenfold.

Genome Mapping

Norflurazon treatment results in a phenocopy of *ghost*, a mutation mapped to chromosome 11 (Tanksley et al., 1992). To determine whether this mutant corresponds to a lesion in *PDS*, we mapped this gene in the tomato genome. Hybridization of *PDS* probes to genomic DNAs revealed an XbaI polymorphism between *Lycopersicon esculentum* and *L. penelii* (K. Alpert, personal communication). We scored this polymorphism in a population of 67 F_2 individuals from a *L. esculentum* × *L.*

Table 2. Light Regulation of *PSY* and *PDS* Expression

Light Treatment	Relative Expression[a]	
	PSY	*PDS*
Deetiolation		
0 hr	0.21	0.45
4 hr	0.26	0.61
24 hr	0.31	0.54
Dark adaptation		
0 day	1.00	1.00
1 day	0.14	1.02
3 days	0.15	0.47

[a] Values normalized as described in Methods.

penelii cross. Segregation of the corresponding alleles showed no significant deviation from the expected 1:2:1 ratio (χ^2 = 2.16). Analysis of the linkage data indicates that *PDS* maps to chromosome 3, at ~10.2 and 14.5 centimorgans from markers TG94 and TG152, respectively (Tanksley et al., 1992). Therefore, *PDS* does not correspond to the *GHOST* locus.

Expression in Developing Leaves, Flowers, and Fruits

Leaves

We analyzed *PSY* and *PDS* expression during stages of leaf development in which there is cell division, cell elongation, chloroplast maturation, and an increase in the number of chloroplasts per cell. The stages of leaves, flowers, and fruits used in this work are shown in Figure 4. Between stage 1 (approximate length of 35 mm) and stage 4 (approximate length of 140 mm), *PSY* mRNA levels decline slightly, whereas *PDS* expression increases more than threefold (Table 3).

Flowers

During flower development, a gradual conversion of chloroplasts into chromoplasts results in mature flowers that contain chloroplasts only in sepals and carpels. In petals, this process results in a greater than sevenfold decline in chlorophyll content and in a greater than eightfold increase in carotenoids (Table 1). During early (1 and 2) stages of flower development, *PSY* expression is more than twofold higher than in mature leaves, whereas *PDS* is expressed at basal levels. A process of induction of both genes begins at stage 3. Expression levels rise further immediately before anthesis (stage 4) to reach levels that at stage 5 are more than ninefold higher than in stage 1 (Table 3).

Fruits

As in flower development, fruit ripening also involves the conversion of chloroplasts into chromoplasts. During fruit ripening, the color of the pericarp changes from green to red as chlorophyll is degraded and carotenoids accumulate (Khudairi, 1972). During this process, chloroplasts differentiate into chromoplasts, chlorophyll content declines to undetectable levels, and carotenoid content increases (Table 1). From immature green to orange, *PSY* is induced more than 25-fold, but *PDS* mRNA levels increase less than threefold (Table 3).

Expression in Organs of Mature Plants

Expression of *PSY* and *PDS* in organs of mature plants is maximal in flowers and fruits and minimal in roots (Table 3). In sepals and ovaries of mature flowers, transcript levels for both genes are similar to leaf levels. Transcript levels in petals, however, are higher than in sepals for both *PDS* (>13-fold) and *PSY* (>25-fold). Levels of mRNA in anthers are also higher than in sepals for both *PDS* (>17-fold) and *PSY* (>sevenfold; Table 3). The difference in mRNA levels between petals and roots is greater than 316-fold for *PSY* and greater than 94-fold for *PDS*. Expression of both genes in stems is slightly lower than in mature leaves.

Table 3. Tissue-Specific Expression of *PSY* and *PDS*

Sample	Stage/Part/ Treatment[a]	Relative Expression[b]	
		PSY	*PDS*
Seedling	Control	1.00	1.00
	Norflurazon	2.02	10.27
	ghost green	1.00	1.00
	ghost white	3.18	5.05
Stem		0.86	0.76
Root		0.11	0.08
Leaf	1	2.40	0.45
	2	2.00	0.71
	3	1.95	0.85
	4	1.60	1.42
	5	1.00	1.00
Flower	1	2.80	0.61
	2	2.85	0.68
	3	8.25	3.98
	4	17.20	7.94
	5	26.45	9.35
	Sepals	1.37	0.54
	Petals	34.80	7.52
	Anthers	10.40	9.50
	Ovaries	0.96	0.56
Fruit	IG	3.03	1.49
	MG	8.20	2.01
	Orange	75.13	4.19
	Red	41.70	0.80

[a] Stages are numbered as given in Figure 4. *ghost* green and white, seedlings from seed of a heterozygous plant harvested as green (genotype +/+ or +/*gh*) and white seedlings (genotype *gh*/*gh*), respectively. IG, immature green; MG, mature green.
[b] Values normalized as described in Methods.

Figure 4. Stages of Development of the Samples of Leaf, Flower, and Fruit Materials Used in This Work.

For leaves and flowers, stages are numbered one through five. Fruit stages are numbered one through four. For the experiments reported in Table 3, whole flowers were used as shown, or individual organs were dissected from stage five flowers. Only pericarp tissue was used for fruit experiments. Scale 1:1.

It is important to note that under the standardization conditions used here, we cannot compare *PSY* with *PDS* mRNA levels. However, screening of a fruit cDNA library with probes for both genes indicated that there are eightfold more *PSY* than *PDS* clones (data not shown), a value that is in general agreement with the results shown here.

DISCUSSION

We have used a sensitive amplification technique to study the expression of *PDS* and *PSY* during plant development and in response to chemical and genetic blocks of the carotenoid biosynthesis pathway.

Confirming previous results by other groups (Pecker et al., 1992; Ray et al., 1992), we were unable to detect either *PDS* or *PSY* transcripts by blot analyses of 30 μg of leaf RNA. Using the more sensitive amplification technique, we determined that both genes are expressed at levels higher than in roots in all green tissues tested (leaves, stem, sepals, and green fruit). We were able to detect both *PDS* and *PSY* transcripts with as little as 3 ng of leaf RNA, indicating that our RT-PCR procedure is greater than 10^4-fold more sensitive than RNA blots.

Light and Carotenoid Biosynthesis

Carotenoids function as photoprotectants in harmful photochemical reactions catalyzed by chlorophyll. Angiosperms follow the preventive strategy of producing the photoprotectants before light activates the production of the sensitizer. In agreement with the observation that carotenoids are made in the dark, our results indicate that deetiolation does not induce the expression of *PSY* and *PDS*. Light induces the synthesis of light-harvesting proteins that bind carotenoids. Thus, the increase in carotenoid content during deetiolation could be due at least partially to the increase in available binding sites.

We report that blocks in carotenoid biosynthesis induced by norflurazon treatment or by the *ghost* mutation induce *PDS* expression and, to a lesser extent, *PSY* expression. This induction could be due either to photooxidative stress or, as in fungi (Bejarano and Cerdá-Olmedo, 1989), to end-product regulation of carotenogenesis.

Photooxidative stress depresses the transcription of photosynthesis-related genes in tomato seedlings lacking colored carotenoids (Giuliano and Scolnik, 1988). This is consistent with observations that in both monocots and dicots optimal rates of transcription of nuclear genes encoding plastid proteins require plastid integrity (Taylor, 1989). Several lines of evidence indicate that maximum expression of nuclear genes encoding plastid proteins may require a "plastidic factor." This factor would no longer be produced under photooxidative conditions, thus resulting in a decline in the transcription of photosynthesis-related nuclear genes (Taylor, 1989). However, expression of *PDS* and *PSY* (this work) and of the spinach gene for the 28-kD chloroplast RNA binding protein (Tonkyn et al., 1992) proceeds under photooxidative conditions. Thus, expression of a subset of nuclear genes for plastid proteins does not require plastid integrity or the putative plastidic factor.

A recent report (Adamska et al., 1992) indicates that the synthesis of early light-inducible proteins (ELIPs) is induced by high light intensity, which can also result in photooxidative damage. Because ELIPs may bind carotenoids (Lers et al., 1991), an increase in their abundance may also raise carotenoid levels in plastid membranes. Thus, the plant response to photooxidative stress involves the negative regulation of nuclear (Taylor, 1989) and plastidic genes (Sagar and Briggs, 1990; Tonkyn et al., 1992) encoding proteins that bind chlorophyll, the photosensitizer, and it may entail the positive regulation of genes encoding proteins for the biosynthesis and accumulation of carotenoids, the photoprotectants.

The *ghost* Mutant

The *ghost* mutant is of particular interest because of its epigenetic variegation effect, which results from changes in carotenoid biosynthesis. Phytoene accumulates in white *ghost* sectors, whereas green sectors have wild-type carotenoids (Rick et al., 1959; Scolnik et al., 1987). We have now mapped *PDS* to chromosome 3 in the tomato genome. Thus, this gene does not correspond to the *GHOST* locus, which controls the desaturation of phytoene. At present we have no further information on the nature of *GHOST*. Genomic gel blot analyses (B. Aracri, G. E. Bartley, P. A. Scolnik, and G. Giuliano, manuscript in preparation) and the mapping results indicate that *PDS* is a single copy gene. The plant *PDS* genes sequenced so far are highly conserved (>79% nucleotide identity between the soybean and tomato sequences encoding the mature protein; B. Aracri, G. E. Bartley, P. A. Scolnik, and G. Giuliano, unpublished data). Thus, it seems unlikely that *GHOST* is a second copy of *PDS*. *GHOST* could encode an ancillary factor needed for Pds activity. If this is the case, this factor must also exist in bacteria because plant *PDS* genes encode active proteins in both *Rhodobacter capsulatus* (Bartley et al., 1991b) and *Escherichia coli* (Pecker et al., 1992). Finally, we must consider the possibility that *GHOST* is not directly related to carotenoid biosynthesis but that it encodes a product necessary for normal plastid development, including pigment accumulation.

Expression in Flowers and Fruits

Chromoplasts of tomato flowers and fruits differ in both the structure of the internal membranes and the carotenoid composition. Yellow carotenoids (xanthophylls) color petals and anthers, whereas fruit chromoplasts are red, mostly due to the presence of lycopene. Our results indicate that, regardless of

pigment composition, the highest levels of *PSY* and *PDS* expression are found in tissues that contain chromoplasts.

We observed a differential regulation of *PSY* and *PDS* during chromoplast development in flowers and fruits. Expression of both genes increases ~10-fold during the stages of flower development tested. However, in orange fruits, expression of *PSY* is more than 25-fold higher than in immature green fruits, but *PDS* expression increases only threefold. The physiological basis for this difference may be the timing of chromoplast development in flowers and fruits. As shown in Table 1, 82% of the total increase in carotenoid content during flower development takes place at stage 4, which precedes anthesis by only a few hours. Thus, this burst in carotenoid biosynthesis may require the coordinate induction of *PSY*, *PDS*, and possibly of other genes encoding different components of the pathway. In contrast, chromoplast fruit development is a relatively slow process (Khudairi, 1972).

Proteins that bind carotenoids are likely to contribute significantly to the accumulation of these pigments in plastids. Besides the regulation of ELIPs discussed above, the levels of a protein that binds carotenoids in pepper fruits increase during ripening (Cervantes-Cervantes et al., 1990).

Using dot blots containing total RNA, Maunders et al. (1987) determined that expression of *PSY* (pTOM5) increases during tomato fruit ripening. Pecker et al. (1992) had to use 25 μg of poly(A)$^+$ RNA from tomato fruits to detect a *PDS* transcript, which appears to increase more than 10-fold during ripening. This value is considerably higher than the one reported here. This discrepancy may be due to unequal loading of poly(A)$^+$ RNA, a possibility not contemplated in that report. Alternatively, this difference could be due to environmental conditions and/or to the genetic backgrounds of the tomato lines used.

The work reported here supports a model of control of carotenoid gene expression by photooxidative stress and plastid development. Tomato *PSY* and *PDS* provide an important tool to elucidate the molecular basis of these control mechanisms.

METHODS

Plant Material

Leaf, floral, and fruit tissues were harvested between 10 and 12 AM from 3-month-old tomato (*Lycopersicon esculentum*) plants (cv Rheilands Rhum) grown in the greenhouse at 22 to 25°C. For light regulation experiments, seeds were sterilized for 20 min in a 20% solution of commercial bleach and germinated aseptically for 7 days at 20°C in half-strength Murashige-Skoog medium (Murashige and Skoog, 1962) under continuous light (~4000 lux, Osram warm-tone fluorescent lamps) or continuous darkness. After the various light/dark shifts, the upper half of the hypocotyl and the cotyledons were harvested under a green safelight. For *ghost* seedlings, seeds harvested from a heterozygous (*gh*/+) plant were sterilized and grown aseptically. White (*gh*/*gh*) and green (+/*.*) seedlings were harvested separately. Carotenoid and chlorophyll concentrations were determined according to the method of Lichtenthaler (1987).

RNA Extraction

Approximately 0.2 g of tissue was ground with an Ultra-Turrax homogenizer set at top speed in 0.5 mL of buffer A (4 M guanidine thiocyanate, 1% sarcosyl, 0.1 M sodium acetate, pH 5.2, 0.7% 2-mercaptoethanol) premixed with 0.4 mL of water-equilibrated phenol. After homogenization, 0.1 mL of chloroform was added, and the mixture was vortexed, cooled on ice, and centrifuged for 5 min at 15,000*g*. The upper phase (0.6 mL) was precipitated with 1 volume of isopropanol. The RNA was collected by centrifugation, resuspended in 100 μL of diethylpyrocarbonate-treated water, and further purified by adding 20 μL of ethanol on ice, collecting the precipitate (mostly polysaccharides) by centrifugation, and precipitating the RNA from the supernatant with 0.33 volume of lithium chloride (8 M) on ice for 30 min. After centrifugation, the RNA pellet was washed with 80% ethanol, resuspended in water (50 μL), and quantified spectrophotometrically. Typically, the OD_{260}-to-OD_{280} ratio was between 1.85 and 1.95. RNA was diluted to 100 μg/mL, and its integrity was checked on formaldehyde gels.

Control Transcript

pBluescript SK+ (2 μg; Stratagene) was linearized with ScaI and transcribed in vitro with T3 or T7 RNA polymerase according to the manufacturer's directions (Boehringer Mannheim). After treatment with 1 unit per μg plasmid of DNase RQ1 (Promega), the mixture was extracted with phenol/chloroform and precipitated with ethanol; the transcript was resuspended in diethylpyrocarbonate-treated water, quantified spectrophotometrically, and its integrity was determined in formaldehyde gels. To ensure that no undegraded plasmid DNA was left in the control transcript, dilutions of the T3 and the T7 transcripts were subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) with primers specific for the T3 transcript (see below), and only T3 transcripts that gave signals more than 100-fold higher than T7 transcripts were used in further experiments.

RT-PCR

RT-PCR was performed using the RNA PCR kit (Perkin Elmer-Cetus) according to the manufacturer's instructions with the following modifications: the reverse transcriptase reaction was made up in a volume of 5 μL in the presence of 100 fg of control transcript, varying amounts (1 to 100 ng) of plant RNA, 50 ng each of downstream primers for both the transcript under study and the control transcript, dithiothreitol (0.5 mM), and molecular biology grade bovine serum albumin (Boehringer Mannheim, 100 μg/mL). The reaction was overlayed with mineral oil (Sigma). After 30 min at 42°C, followed by 5 min at 99°C to inactivate the reverse transcriptase, the tubes were transferred to ice. Twenty microliters of PCR master mix containing 50 ng of upstream primers for both the gene under study and the control transcript and 0.6 units *Taq* polymerase was added to each sample. After brief spinning to mix contents on the bottom of the tube, the samples were amplified with the following protocol: 94°C (2 min), then 28 cycles at 93°C (1 min), 55°C (1 min), and 72°C (1 min) in a DNA Thermal Cycler (Perkin Elmer-Cetus). Due to the lack of thermal homogeneity among different rows of the instrument, only samples amplified in the same row were compared.

The following oligonucleotides were used in amplifications: *PDS*, GGCACTCAACTTTATAAACC (upstream) and GATTGGGGTTGTAATATTCC (downstream); *PSY*, CAGCCTTAGATAGGTGGGAA (upstream)

and GCCTTTCTCTGCCTCATCAA (downstream); control transcript, GTAGCCTGAATGGCGAATGG (upstream) and ACGTGGACTCCAACGTCAAAG (downstream).

Quantitation of RT-PCR Products

A 12-μL aliquot of the reaction was loaded on a 1.5% agarose gel containing 1 × TBE and 0.2 μg/mL ethidium bromide and electrophoresed for 30 min at 5 V/cm. The gel was photographed using Polaroid Type 55 (positive-negative) film. The intensity of the bands was quantified by scanning the negatives on a spectrophotometer (model DU-70; Beckman) equipped with a gel scanning device. Only bands having an optical density of 0.05 to 0.25 were utilized to calculate RNA abundances. Relative values were obtained by comparison to a reaction containing 10 ng of leaf RNA and normalized for sample-to-sample variation in RT and PCR efficiency by comparing the intensities of the bands given by the control transcript.

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