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Comparison of Carotenoid Content, Gene Expression and Enzyme Levels in Tomato (*Lycopersicon esculentum*) Leaves

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Physiological conditions which lead to changes in total carotenoid content in tomato plantlets were identified. Carotenoid levels were found to increase after the onset of a dark period during a normal 24h cycle. This rapid initial increase is followed by a steady decrease in carotenoid content throughout the night. A decrease in the expression of several carotenogenic genes, namely *pds*, *zds* (carotenoid desaturases) and *ptox* (plastid terminal oxidase), was observed following the removal of the light (when carotenoid content is at its highest). An increase in gene expression was observed before the return to light for *pds* and *zds* (when carotenoid levels were at their lowest), or following the return to light for *ptox*. The phytoene desaturation inhibitor norflurazon leads to a decrease coloured carotenoid content and, in the light, this correlated with *pds* and *zds* gene induction. In the dark, norflurazon treatment led to only a weak decrease in carotenoid content and only a small increase in *pds* and *zds* gene expression. The striking absence of phytoene accumulation under norflurazon treatment in the dark suggests a down-regulation of carotenoid formation in darkness. However, prolonged dark conditions, or treatment with photosynthetic inhibitors, surprisingly led to higher carotenoid levels, which correlated with decreased expression of most examined genes. In addition to light, which acts in a complex way on carotenoid accumulation and gene expression, our results are best explained by a regulatory effect of carotenoid levels on the expression of several biosynthetic genes. In addition, monitoring of protein amounts for phytoene desaturase and plastid terminal oxidase (which sometimes do not correlate with gene expression) indicate an even more complex regulatory pattern.

Key words: Carotenoid Desaturation, Photooxidation, Norflurazon

Introduction

In photosynthetic tissues carotenoids are synthesized and located in the chloroplasts where they accumulate primarily in the photosynthetic membranes in association with the light harvesting complex and reaction centres (Cunningham and Grant, 1998). Carotenoids have a variety of biological functions which include stabilization of lipid membranes (Havaux, 1998), light harvesting for photosynthesis and protection against photooxidation (Frank and Cogdell, 1996). The protective functions of carotenoids associated with the reaction centres and antenna complex are so critical that an inability to form cyclic carotenoids, due to a block in carotenoid biosynthesis by a herbicide such as norflurazon (Simkin *et al*., 2000) or by a mutation such as that observed in the tomato (*Lycopersicun esculentum*) ghost mutation (Josse *et al*., 2000), is eventually lethal in oxygen-evolving photosynthetic organisms (Sandmann and Böger, 1989).

Tomato contains 2 genes for phytoene synthase (*psy-1* and *psy-2*) which catalyzes the first committed step in carotenoid biosynthesis. Although these two enzymes are 95% identical, antisense inhibition of *psy*-1 inhibited carotenoid accumulation in fruit only (Bramley *et al.*, 1992) indicating that *psy-2* is likely to be the most important one

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorodiphenyl)-1,1-dimethyl urea; NF, norflurazon (4-chloro-5-methylamino-N-[*m*-trifluormethylphenyl] pyridazinone); CN, control; RT-PCR, Reverse transcription polymerase chain reaction.

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for carotenoid biosynthesis in green tissues. It was reported (Bartley and Scolnik, 1993) that *psy-1, psy-2,* but not *pds* (*encoding* phytoene desaturase), were up-regulated in tomato seedlings grown in the light *vs.* darkness. Phytoene desaturation to lycopene is catalyzed by phytoene desaturase followed by ζ-carotene desaturase (encoded by *zds*) and requires the presence of a plastid terminal oxidase (encoded by *ptox*) as a cofactor (Carol *et al.*, 1999; Carol and Kuntz, 2001; Josse *et al.*, 2000). Lycopene is subsequently converted to cyclic carotenoids by cyclases such as lycopene β-cyclase (*lcy-b*; Pecker *et al.*, 1996) and lycopene ε-cyclase (Ronen *et al.*, 1999).

In this report, tomato plantlets were used to address 3 questions: i) are the various carotenoid biosynthetic genes co-expressed in leaves, as observed during fruit ripening for *pds, zds* and *ptox* (Josse *et al.*, 2000) and ii) how does mRNA level correlate with the level of the encoded polypeptides; iii) is there a link between gene expression and carotenoid content. Conditions which trigger changes in carotenoid content were identified and used as an experimental basis to address these questions. Our data point to the existence of complex and intricate regulatory mechanisms controlling gene expression, which implicate light and carotenoid content.

Results

Diurnal changes in carotenoid biosynthesis in tomato leaves

In a first set of experiments (Fig. 1a), the concentration of total carotenoids was determined in tomato leaves during a 24-h cycle. A steady state level was found during the light period. At the onset of darkness, the levels of total carotenoids increase rapidly and then gradually decrease during the night. Under our experimental conditions, they reach a level below that in the light. Following the return of the light after 8 hours of darkness [from 10 pm to 6 am], the carotenoid levels transiently increased above the normal level of the light period. This increase is also observed if the plants are not returned to the light (data not shown). In the light, the carotenoid content then rapidly returns to the steady state level. In plants maintained in the dark, the carotenoid content remains slightly elevated (data not shown).

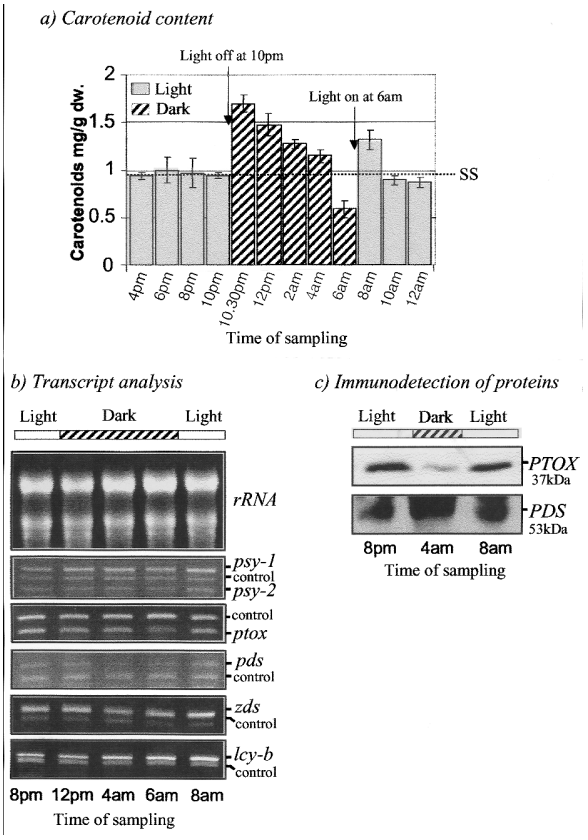

Fig. 1. Diurnal change in carotenoid biosynthesis over a 24-h period.
(a) Carotenoid content (mg/g dw) in the plants used for RT-PCR are shown. SS indicates the steady state level during the daylight period.
(b) Diurnal regulation of gene expression studied by comparative RT-PCR. The expression of *psy-1, psy-2, ptox, pds, zds* and *lcy-b* genes were monitored in the same leaves used for carotenoid analysis. PCR products were separated by 1.5% agarose gel electrophoresis and visualised by ethidium bromide staining. Equal amounts of total RNA (5 ng/μl reaction) were used in each reaction. Amplification of globin mRNA (added to RT reaction mix) was used as a control for the RT-PCR reaction.
(c) Diurnal regulation of protein levels by Western blotting. Chloroplast proteins were separated on 12.5% polyacrylamide gels. Results were visualised by immunodetection with the relevant antibodies (PTOX, PDS) following transfer to immobilon-P membrane.

These observations (i.e. fluctuating carotenoid levels) prompted us to examine gene expression levels. Transcripts levels were compared by RT-PCR (Fig. 1b) in the same plant samples used for carotenoid analysis. RT-PCR was selected since it

has proven reliable in our own work, with this material and genes (Simkin *et al.,* 2000, Josse *et al.,* 2000). Transcript levels of *pds* and *zds* were down-regulated during the night period, by >8 and 2 fold respectively, dropping to the lowest level at 4 am (6 hours after the removal of light). However, at 6 am (immediately before the return of light) both *pds* and *zds* transcripts were elevated to a level above that observed at 4 am. The transcript level of *ptox* decreased progressively during the night until a 3 fold decrease was observed at 6 am, but in this case it re-increased later (following the return of the light; Fig. 1b). In contrast, *lcy-b* transcript levels increased during the night period whilst *psy-1* and *psy-2* transcript levels were relatively stable.

To determine the relationship between transcript levels and enzyme amounts, chloroplasts were extracted from plant samples treated under the same conditions and enzyme amounts compared by immunodetection (when antibodies were available). Clearly lower PTOX polypeptide (37 kDa) levels were found during the night (4 am, Fig. 1c) indicating that changes in mRNA levels during the night are accompanied by even greater decrease in protein amount. In comparison, PDS polypeptide (53 kDa) levels were not found to decrease (Fig. 1c) in sharp contrast to its decreasing mRNA levels.

Effects of an inhibitor of carotenoid biosynthesis

Norflurazon (NF), a phytoene desaturase inhibitor (Böger and Sandmann, 1990; Sandmann and Albrecht, 1990; Steiger *et al*., 1999) was used to design additional experimental conditions caracterized by altered carotenoid content. First, tomato plants were treated for 48 hours in parallel with control plants. For the purpose of this experiment we chose only the 2 leaves at the top of the plant which showed the greatest loss of chlorophyll and compared them to the corresponding leaves from the control plant. It should be mentioned that these samples were therefore slightly different from the ones of the diurnal experiments (where several larger leaves were pooled and analyzed) which likely explains why these leaves showed a different total carotenoid content between both sets of experiments. This type of variation was not further studied in the present work.

As expected, tomato plantlets treated with NF in light showed an accumulation of phytoene (Table IA) and a decrease in other (coloured) carotenoids. However the total amount of carotenoids (precursors plus other carotenoids) in the NF-treated plants is higher than seen in the control plants suggesting an up-regulation of this biosynthetic pathway. An increase of 18% ±8% was observed under strong light conditions (Table IA). An even greater increase, 49% ±7%, is seen under normal light conditions where photooxidation is reduced (Table IA). RT-PCR experiments (Fig. 2) indicated an increase in *pds* and *zds* transcripts levels by 8 and 3.5 times, respectively (Fig. 2a) upon NF treatment in the light. In reasonable agreement with results from Giuliano *et al.* (1993); Bartley and Scolnik (1993) who reported only marginal changes in *psy1/2* expression in tomato seedlings upon NF treatment, we observed no clear difference in *psy-1* or *psy-2* transcript in any experiment (note that the apparent difference in Fig. 2a for these genes is paralleled by the same changes in the standard transcript). In addition, no increases in *lcy-b* or *ptox* transcript level were observed.

Immunodetection experiments (Fig. 2b) with chloroplast protein extracts from plants treated with NF under the same conditions indicated an increase in PDS polypeptide (53 kDa) level by a factor of 5, indicating that the increase in its tran-

Table I. Carotenoids (mg/g dw) in leaf samples treated with inhibitors for 48 hours.

	Phytoene	Coloured Carotenoids	Total
A.			
Normal Light	0	1.98 (± 0.10)	1.98
+NF	1.32 (± 0.04)	1.63 (± 0.09)	2.95
Strong Light	0	1.85 (± 0.01)	1.85
+ NF	1.24 (± 0.12)	0.94 (± 0.03)	2.18
Dark	0	2.15 (± 0.03)	2.15
+ NF	0.05 (± 0.02)	2.13 (± 0.02)	2.18
B.			
Strong Light	0	1.34 (± 0.02)	1.34
+ DCMU	0	1.90 (± 0.40)	1.90
+ DBMIB	0	1.43 (± 0.08)	1.43
Dark	0	1.97 (± 0.02)	1.97

Standard deviations are shown in brackets. Values are means of 3 to 4 determinations. Each set of experiments was carried out with several sets of seedlings. Norflurazon (NF, 1 mM), DCMU (4 mM) or DBMIB (4 mM). Phytoene values represent the sum of phytoene and its oxidation products (Sandmann and Albrecht, 1990).

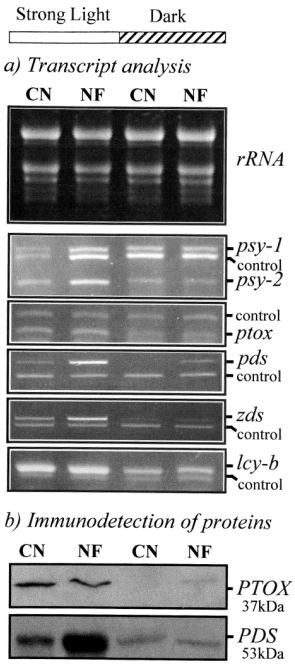

Fig. 2. Analysis of carotenoid biosynthesis after norflurazon treatment.
(a) Gene expression studied by comparative RT-PCR in tomato plants in the light and dark. The expression of *psy-1, psy-2, ptox, pds, zds* and *lcy-b* genes in the leaves of tomato plantlets were monitored.
(b) Chloroplast protein levels were studied by Western blot. Total proteins were separated on 12.5% polyacrylamide gels. Results were visualised by immunodetection with the relevant antibodies (PTOX, PDS) following transfer to immobilon-P membrane. CN: non-treated control; NF: norflurazon; ▭: light; ▨: dark.

script level is accompanied by an increase in protein levels. No increase in PTOX polypeptide (37 kDa) levels were observed in agreement with the observed unchanged *ptox* transcript levels (Fig. 2b).

When tomato plants were treated for 48 h with NF in the dark, accumulation of phytoene was very low (Table IA), which seems to indicate a reduced *de novo* synthesis of carotenoids. On average the levels of coloured carotenoid were similar in NF treated and control plants in the dark (both slightly higher than in the light exposed plants, Table IA), although slightly lower levels were occasionally observed in NF treated plants. It should be mentioned that our experimental growth conditions included a circulation of air in order to avoid that plants in the light had higher transpiration rate than plants in the dark. Therefore, we assume that NF uptake was similar under both conditions.

The RT-PCR results showed that the transcripts of *psy-1, lcy-b* and *ptox* in the dark are subjected to a marginal (ca. 2 fold) down-regulation in comparison with the light samples. *zds* and *pds* showed a 3 and >6 fold decrease, respectively, in transcript levels in the dark, whilst for *psy-2*, the most important for carotenoid biosynthesis in green tissue, Bartley and Scolnik (1993) showed an 8 fold decrease. Compared to these untreated dark controls, plantlets treated in the dark with NF exhibited an increase in *pds* and, to some extent, in *zds* transcript levels. It should be emphasized that these plants being in the dark were not exposed to photooxidation and did not differ in detectable phytoene accumulation, but slightly in their content of coloured carotenoids.

In the dark-incubated tomato plants, PTOX polypeptide levels were found to decrease during the prolonged dark period (Fig. 2b) indicating that changes in mRNA levels during prolonged dark period are accompanied by even greater decrease in protein amount. This result is consistent with that observed during the diurnal dark period (Fig. 1c). PDS polypeptide levels were also found to decrease during prolonged darkness (Fig. 2c).

Effects of photosynthetic inhibitors on carotenoid content and gene expression.

We also examined the effect of another herbicide, namely DCMU (Brusslan and Haselkorn, 1989), which inhibits photosynthetic electron flow at the level of PS-II. Application of this herbicide does not lead to an immediate bleaching effect. However, we observed after 48 h of DCMU treatment in the light a high increase in total carotenoid content (Table IB). It should be mentioned that this increase in carotenoid content varied between samples (Table IB). The link between the photosynthetic inhibitory effect of DCMU and carotenoid content is currently unclear. This is reminiscent of the effect of prolonged darkness also in the fact that, in the presence of DCMU, NF treatment in the light does not lead to substantial phytoene accumulation (Table IB). It is interesting to observe that the DCMU-triggered increase in

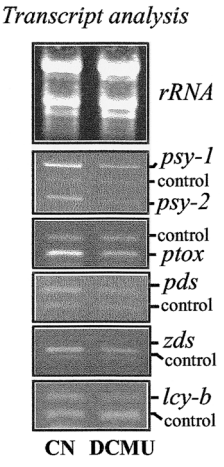

Fig. 3. Gene expression studied by comparative RT-PCR in plants treated with DCMU in the light. The expression of *psy-1, psy-2, ptox, pds, zds* and *lcy-b* genes in the leaves of tomato plantlets were monitored. Equal amounts of total RNA (5ng/μl reaction) were used in each reaction. CN: non-treated control; DCMU.

carotenoid concentration is accompanied by a 2 fold decrease in the transcript levels of *psy-1, pds, zds,* and *lcy-b* and a 4 fold decrease of *psy-2* and *ptox* (Fig. 3). However, this decrease is not as strong as seen in the samples treated in the dark for a period of 48 h.

An increase in carotenoid content (Table IB) and a corresponding decrease in gene expression (not shown) were also observed upon treatment with DBMIB. The latter compound also blocks photosynthetic electron flow, this time at the level of the cytochrome *b*6/*f* complex. In theory, DCMU and DBMIB treatment trigger opposite effects on the redox state of the plastoquinone pool, which can lead to differential expression of nuclear genes involved in photosynthesis (Escoubas *et al.,* 1995). It should be stressed that DBMIB may have more complex effects when applied to whole plants. It is therefore not possible to draw any conclusion regarding the redox state of the plastoquinone pool from the present study. However, it should be noted (Table II) that DBMIB, as DCMU, prevents phytoene accumulation upon norflurazon treatment in the light.

Table II. Carotenoids (mg/g dw) in leaf samples treated with inhibitors norflurazon (NF, 1 mM), NF + DCMU (4 mM) or NF + DBMIB (4 mM).

	Phytoene	Coloured Carotenoids	Total
Strong Light	0	1.35	1.35
+ NF	0.40	0.93	1.33
+ NF + DCMU	0.08	1.76	1.84
+ NF + DBMIB	0.04	1.52	1.56

Standard deviations are in the range of 4–9%. Values are means of 3 to 4 determinations. Phytoene values represent the sum of phytoene and its oxidation products (Sandmann and Albrecht, 1990).

Discussion

In this report, we used various experimental conditions found to trigger important changes in carotenoid levels in tomato plantlets. First, under a normal light/dark regime, the constant steady state levels observed during most of the day (most likely as a result of constant synthesis and degradation under growth chamber conditions) contrasts with a transient increase at the beginning of a dark period. This may possibly be explained by a level of biosynthesis transiently exceeding the level of turnover due to a sudden absence of light-dependent turnover. This explanation is in line with our observation that a similar increase in carotenoid content occurs when the plants are placed into the dark 4 h before the onset of the normal dark period (data not shown). Our experimental conditions (sudden removal of the light) may allow us to monitor a phenomenon which may otherwise, under progressive decline in light intensities, remain weaker. This increase in carotenoid content is followed by a progressive decrease which may be due to reduced synthesis in darkness and light independent metabolic cleavage of carotenoids. Thus the present data suggest that fluctuations in carotenoid content are determined by synthesis, light-dependent and light independent turnover.

Upon the return of light, carotenoid levels showed a transient peak which is best explained by a strong induction of their biosynthesis and a delay in the operation of the light-dependent turnover. When plants are kept in darkness for a prolonged period it is surprising that increased carotenoid content was observed (in contrast to the decrease during the night). It should be mentioned that when the return of light is omitted at the end of a normal dark period an increase is also ob-

served (not shown). However, a role of circadian rhythm in the latter phenomenon cannot be ruled out. After 48 hours in the darkness, the carotenoid content remains slightly elevated above that observed in the light (see Table I). One can only speculate that, under conditions of prolonged darkness (in excess of the 8 hour diurnal period), light-independent metabolic cleavage of carotenoid is minimal.

We also observed that inhibition of carotenoid desaturation by NF does not lead to phytoene accumulation in the dark. This can be explained by a reduced flux into the carotenogenic pathway and would be in line with the above mentioned hypothesis that a strongly reduced carotenoid synthesis occurs in darkness. Although the NF concentration employed was 1 mM which is more than 1000-fold higher than the I_{50} value for this herbicide (Simkin *et al.*, 2000), it also seems possible that the desaturase activity may not be 100% blocked. Both explanations are not mutually exclusive: the low level of phytoene synthesized in darkness may be readily converted by the non-inhibited fraction of the desaturase. This could contribute to maintain carotenoids to levels similar to those of untreated plants. In the light, this may explain why the decline in coloured carotenoid is not dramatic upon NF treatment (a fraction of the accumulating phytoene may eventually become desaturated). Absence of phytoene accumulation upon NF treatment in the light is also observed in presence of the photosynthetic inhibitors DCMU or DBMIB, pointing again, like in darkness, to reduced biosynthetic rates. When applied alone in the light, these inhibitors also mimicked to some extent the effect of prolonged darkness on carotenoid levels (slightly higher coloured carotenoid content; Table II).

These (to some extent unexpected) changes in carotenoid content provide a useful experimental frame to examine transcript levels for carotenoid biosynthetic genes. It appears that all our results can be interpreted by the hypothesis that carotenoid content influences gene expression, at least of *pds, zds* and sometimes *ptox*. They are consistent in showing that high carotenoid levels are accompanied by low expression levels of these genes. This makes sense only if one postulates that carotenoid levels rapidly influence gene expression, while changes in gene expression are not the sole parameter which can in turn control carotenoid levels. These levels are influenced by metabolic turnover with a substantial contribution of photooxidation, as well as other putative regulatory mechanisms acting on synthesis (see below). Our hypothesis that carotenoid content influences gene expression is also corroborated by the fact that reduced coloured carotenoid levels are accompanied by higher gene expression levels (at least for *pds* and *zds*). This is the case upon NF treatment in the light and at the end of a normal dark period when an induction of *pds* and *zds* gene expression was observed before the return of light. In the latter case, however, the persistence of a circadian rhythm may explain the observed re-induction of gene expression.

Our conclusion on a regulatory influence of carotenoid content can also be compared to data published by others. Corona *et al*. (1996) proposed that the induction of the *pds* promoter was responding to end product regulation and that β-carotene and xanthophylls may be candidate compounds. Metabolic engineering of carotenoid content in transgenic plants also suggest that feedback mechanisms occur (Römer *et al*., 2000; al-Babili *et al.*, 2001).

We confirm and extend the results of Giuliano *et al.* (1993) who showed a 2 and 10 fold increase in *psy* and *pds* mRNA levels, respectively, in tomato seedlings treated with NF in the light. It is striking that these data using tomato differ from those obtained using *Arabidopsis* (Wetzel and Rodermel, 1998) and pepper (Simkin *et al*., 2000). In the latter case, these experiments (performed strictly under the same conditions than the one reported here) indicated no significant difference in *zds, pds, ptox* or *psy* transcript levels between leaves of pepper plantlets treated with NF in the light after 48 h and control plantlets. Furthermore, unchanged transcript levels for *psy-1*, *psy-2, zds, pds and lcy-b* were also detected in transgenic tobacco plants with inactivated phytoene desaturase by an antisense approach although an accumulation of phytoene was observed (Busch *et al*., 2002). The reason for these species to species differences is unclear but, since regulation of carotenogenesis is obviously controlled by several regulatory mechanisms, one can propose that some of these mechanisms are prevalent over others in some plants.

Examples of such alternative regulatory factors include light. von Lintig *et al.* (1997) demonstrated that *psy* expression is up-regulated in *Arabidopsis thaliana* seedlings following treatment with continuous far-red and red light. In tomato leaves, light regulation of *psy* mRNA has been reported by various authors (Bartley and Scolnik, 1993; Giuliano *et al.*, 1993). However, in this study we did not see any increase in *psy-1* or *psy-2* mRNA levels in plantlets following exposure to white light, for a period of 30 min, during the dark period (data not shown). Activation of phytoene synthase is a key step in carotenoid biosynthesis during photomorphogenesis. Our data using inhibitors and transcript determinations demonstrate a down-regulation of carotenoid biosynthesis, at the level of phytoene synthase, upon darkness indicating that phytoene synthase is an important regulatory step in carotenoid biosynthesis during light/dark transition.

Our data showing that not all carotenoid biosynthetic genes are co-expressed suggests that regulation is far more complex than transcriptional control. For example, *pds* and *zds* expression show the same trend but are not strictly parallel, although the encoded enzymes work in tandem. *ptox*, which encodes a co-factor for both enzymes, shows a strikingly different expression pattern upon NF treatment in the light.

When enzyme amounts of PDS and PTOX, for which antisera were available, were determined we observed a certain correlation between changes in transcript levels and changes in enzyme levels. One exception is the constant amount of PDS during the night while its mRNA declines. This could be explained by a relative stability of the protein. However, during a longer dark period a decrease in PDS level was observed which is explained by the long period absence of the corresponding mRNA. In general, changes in enzyme amount were not strictly parallel to the changes in the corresponding mRNA amounts. This is not totally unexpected, since turnover rates of mRNA and proteins may be quite different.

Materials and Methods

Plant materials

Tomato (*L. esculentum,* cv. Ailsa Craig, purchased from Seeds-by-size, Hemel Hempstead, UK) plantlets were grown under culture room conditions on soil in 4 cm diameter pots. Plantlets were kept at culture room temperature (24–26 °C) with a 16–8 hour photo-period (light: 150 $\mu E \cdot m^{-2} \cdot s^{-1}$; dark: 0.06 $\mu E \cdot m^{-2} \cdot s^{-1}$). A circulation of air was maintained around the plants by electric fan in order to equalize temperature and evaporation (Simkin *et al.*, 2000). For diurnal experiments tomato plantlets were grown until approximately 15 cm in height. Leaf samples were taken 6 h before the night period (4 pm) and at 2 h intervals for a period of 20 h (until 12 am). Material was immediately frozen in liquid nitrogen and stored at –80 °C. Several leaves from the top part of the plant (growing tip) were taken for analysis. All leaf material used was at the same developmental stage. 4 sets of plants were analyzed using leaves at the same developmental stage in each experiment.

Inhibitor treatment

Tomato plantlets, approx. 10 cm in height, were treated with water or water containing 1 mM norflurazon (NF), 4 mM 3-(3,4-dichlorodiphenyl)-1,1-dimethyl urea (DCMU; Sigma) or 4 mM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB; Sigma) . In a first set of experiments (Table I), plants were kept in normal light (150 $\mu E \cdot m^{-2} \cdot s^{-1}$), or transferred either to stronger light (280 $\mu E \cdot m^{-2} \cdot s^{-1}$) or darkness (0.06 $\mu E \cdot m^{-2} \cdot s^{-1}$) The two top leaves were harvested after 48 h. This time period was selected to obtain a sufficient loss of coloured carotenoids and chlorophyll (in the presence of NF, symptoms of photo-bleaching became apparent in normal light, whilst in high-light leaves showed a 50% loss of coloured carotenoids). An 48-h period was also selected for the DCMU and DBMIB treated samples to maintain a consistent time period with that used for NF treatment. The DCMU or DBMIB-treated plantlets showed a paler green colour but no bleaching. The corresponding leaves were taken from the control plants. In a second set of experiments (Table II), plants were placed into the dark for 16 hours in the presence of these inhibitors (DCMU or DBMIB) and NF. This period was selected to give the plants an efficient period to absorb the inhibitors and NF without deleterious effects. After 16 h the plants were placed into strong light (280 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 8 h and the carotenoid content was determined.

Carotenoid analysis

Frozen plant materials were ground in a mortar. Approximately 10% of the ground plant material was freeze-dried and extracted for 20 min with methanol containing 6% KOH at 60 °C. After partitioning into 10% diethylether in petroleum ether, carotenoids were separated and quantified by HPLC analysis. The system used consisted of a Nucleosil 120-3 C18-column and isocratic elution with acetonitrile/methanol/2-propanol 85:10:5 v/v/v (Breitenbach *et al.*, 1999). Authentic standards were used for identification and quantification of the reaction products. A Kontron (Straubenhard, Germany) diode array detector 440 was used to record the spectra from the elution peaks.

Extraction of total RNA

The remaining frozen ground material was added to 1 ml extraction buffer, 0.1 M Tris (Tris-(hydroxymethyl)aminomethane) pH 8.0, 10 mM EDTA, 0.1 M LiCl, 1% SDS (sodium dodecyl sulfate), mixed with 1 ml water-saturated phenol preheated to 65 °C and vortexed. The samples were centrifuged and the aqueous phase was recovered and re-extracted with 1 ml of chloroform. The aqueous phase was collected upon centrifugation and precipitated overnight with 0.5 volumes 6 M LiCl. Following centrifugation, pellet was washed with 70% ethanol and 100% ethanol, dried and resuspended in RNA resuspension buffer (10 mM Tris pH 7.5, 1 mM EDTA, 1% SDS) and precipitated in 2 volumes absolute ethanol and 0.1 volumes Na acetate. RNA samples were treated with 20 μg/ml Proteinase K in buffer (10 mM Tris pH 7.0, 0.4% SDS) at 50 °C and re-purified by phenol/chloroform extraction. Concentration and purity of total plant RNA was determined by spectrophotometric analysis. All RNA samples in each experiment were analyzed by formaldehyde agarose gel electrophoresis and visual inspection of rRNA bands upon ethidium bromide staining (see Figures 1a–3a). Samples were treated with DNase in 25 μl buffer (20 mM Tris pH 7.0, 6 mM MgCl, 40 u RNase inhibitor (RNaseOUT, BRL), 0.1 u DNaseI) to remove DNA contamination. Samples were checked for DNA contamination by PCR using 3 μg of total RNA and gene specific oligonucleotides. The amplification reaction included 32 cycles of 30 sec at 94 °C, 20 sec at 50 °C and 25 sec at 72 °C.

Measurement of mRNA by RT-PCR

Reverse transcription was carried out using 500 ng of total RNA and oligo-dT as a primer. The reaction mixture included 1 mM dNTPs, 0.5 μM oligo-dT, 20 u RNase inhibitor, 10 pg of control RNA (rabbit globin mRNA from reticulocyte polyribosomes, BRL), 10 mM DTT, 1× RT buffer and 150 u M-MLV reverse transcriptase (BRL) in total volume of 20 μl (Josse *et al.*, 2000; Simkin *et al.*, 2000). Each reaction was carried out in duplicate. The reaction mixture was incubated for 10 min at 20 °C, 35 min at 37 °C and then 15 min at 42 °C. Duplicate samples were pooled to give final volume of 40 μl for PCR. The PCR reaction contained 1.3× Taq polymerase buffer, 5 mM MgCl, 0.30 mM dNTPs, 1.5 u Taq polymerase (BRL) and 10 μl RT reaction mixture (25 ng RNA/μl) in a total volume of 50 μl. Final PCR concentration was 5 ng/μl reaction. Reaction contained 150 ng–500 ng of selected couples of the following primers:
psy1, TTGGGCTTGTTGAGTGAAGC;
psy2, TGTCGTTGCCTTTGATTCAGG;
pds1, TTGTGTTTGCCGCTCCAGTGGATAT;
pds2, GCGCCTTCCATTGAAGCCAAGTAT;
zds1, ATTATTACATTGAGGGACAAGGCT;
zds2, TCATCAGACAAGACTCAACTCATC;
ptox1, GTATTCATGAACATTAGAGATGACG;
ptox2, GTATATACAAGTATAGTTTGTCCGC;
lcy1, CAGAGAGTCGTTGGAATCGGTGG;
lcy2, CATTCTTTATCCTGTAACAAATTGTTGATC.

Primers for the globin RNA (control1, CTGGG CAGGCTGCTGGT; control2, GATCTCAGTGG TATTTGTGAG) were introduced into each RT reaction as control for RT-PCR. RT-PCR was carried out with and without globin primers to exclude potential problems with multiplexing. To ensure linearity of the reaction, the minimum number of cycles needed to visualize the transcripts was first determined (it was found to be 28 to 30 depending on the transcript) and then RT-PCR were performed using increasing RNA concentrations and 30 PCR cycles, as published previously (Simkin *et al.,* 2000). The amplification reactions consisted of 30 sec at 94 °C, 20 sec at 50 °C and 25 sec at 72 °C (Simkin *et al.,* 2000). When the condition for RT-PCR linearity was established, runs were performed and repeated using independently treated samples. PCR prod-

ucts were fractionated on 1.5% agarose gel. Bands were quantified by an analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/. All transcript values for carotenogenic genes were related to the corresponding value of the amplified globin mRNA. Due to high sequence homology, *psy-1* and *psy-2* were amplified using the same primers as previously described (Bartley and Scolnik, 1993). Amplified products were then digested with Hind III to separate *psy-2* from *psy-1* (Bartley and Scolnik, 1993). A digestion period of 12 hours was used to assure total digestion of the PCR product.

Chloroplasts extraction and immunodetection of chloroplast proteins

The extraction was carried out at 4 °C. Ten leaves were cut into pieces and blended in 1× extraction buffer (sodium pyrophosphate 1 mM ($Na_4P_2O_7$); HEPES 50 mM; sorbitol 330 mM; EDTA 2 mM; $MgCl_2$ 1 mM; $MnCl_2$ 1 mM; DTT 1 mM, pH 6,8). The extract was filtered, centrifuged for 10 min at 1500×*g*, re-suspended in extraction buffer, and loaded onto a Percoll gradient. The gradient was then centrifuged for 20 min at 3000×*g*. The intact chloroplasts are recovered from the interface between the 40% and 80% Percoll. Chloroplast protein concentration was determined by the Lowry method (DC Protein Assay, BioRad). 35 μg of each protein sample was fractionated by SDS/polyacrylamide gel electrophoresis and electroblotted onto Immobilon-P nitro-cellulose. Membranes were probed with antibodies from rabbit source. Immunodetection was performed using the horseradish peroxide conjugate substrate kit (Biorad) and the ECL Western blotting kit (Amersham) as recommended by the suppliers.

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