Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase

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Summary

Plant isoprenoids represent a heterogeneous group of compounds which play essential roles not only in growth and development, but also in the interaction of plants with their environment. Higher plants contain two pathways for the biosynthesis of isoprenoids: the mevalonate pathway, located in the cytosol/endoplasmic reticulum, and the recently discovered mevalonate-independent pathway (Rohmer pathway), located in the plastids. In order to evaluate the function of the Rohmer pathway in the regulation of the synthesis of plastidial isoprenoids, we have isolated a tomato cDNA encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), the first enzyme of the pathway. We demonstrate *in vivo* activity and plastid targeting of plant DXS. Expression analysis of the tomato *DXS* gene indicates developmental and organ-specific regulation of mRNA accumulation and a strong correlation with carotenoid synthesis during fruit development. 1-Deoxy-D-xylulose feeding experiments, together with expression analysis of *DXS* and *PSY1* (encoding the fruit-specific isoform of phytoene synthase) in wild-type and *yellow flesh* mutant fruits, indicate that DXS catalyses the first potentially regulatory step in carotenoid biosynthesis during early fruit ripening. Our results change the current view that PSY1 is the only regulatory enzyme in tomato fruit carotenogenesis, and point towards a coordinated role of both DXS and PSY1 in the control of fruit carotenoid synthesis.

Introduction

Isoprenoids are a large group of compounds which derive from a common building unit, isopentenyl pyrophosphate (IPP). Following the discovery of the mevalonate pathway in the 1950s, it was accepted that isoprenoids were synthesized from acetyl-CoA via mevalonic acid and IPP in all organisms, including plants (McGarvey and Croteau, 1995). In many cases, however, experimental data on the biosynthesis of specific isoprenoids in plants and micro-organisms could not be explained from the exclusive operation of the mevalonate pathway (reviewed by Lichtenthaler *et al.*, 1997). A few years ago, an alternative mevalonate-independent pathway for IPP biosynthesis was initially identified in bacteria by Rohmer and collaborators (Rohmer *et al.*, 1993). Evidence has subsequently emerged indicating that both the mevalonate and the Rohmer pathways operate in higher plants (Lichtenthaler, 1999; Rohmer, 1999). Plant isoprenoids synthesized in the cytosol/endoplasmic reticulum (including hormones such as cytokinins and brassinosteroids, phytosterols for membrane biogenesis, phytoalexins for defence against pathogens, and prenyl groups for post-translational modification of proteins), and in the mitochondria (ubiquinone), are formed from mevalonate-derived IPP. Plastid isoprenoids (including hormones such as gibberellins and abscisic acid, photosynthesis-related pigments such as carotenoids and the phytol moiety of chlorophylls, and the side chain of electron carriers such as plastoquinone, phylloquinone K_1, α-tocoquinone, and α-tocopherol) derive from IPP synthesized from the Rohmer pathway (Figure 1).

The initial reaction of the Rohmer pathway involves the formation of 1-deoxy-D-xylulose 5-phosphate (DX5P) by condensation of (hydroxyethyl)thiamin derived from the decarboxylation of pyruvate with the C1 aldehyde group of D-glyceraldehyde 3-phosphate (Arigoni *et al.*, 1997; Rohmer *et al.*, 1996). This reaction is catalysed by

1-deoxy-D-xylulose 5-phosphate synthase (DXS), a novel type of transketolase (Lange *et al.*, 1998; Lois *et al.*, 1998; Sprenger *et al.*, 1997). In the second step, DX5P is converted to 2-*C*-methyl-D-erythritol 4-phosphate (ME4P) by the enzyme DX5P reductoisomerase (Lange and Croteau, 1999; Schwender *et al.*, 1999; Takahashi *et al.*, 1998). The remaining enzymatic reactions leading to the synthesis of IPP have not yet been fully elucidated.

In plants, it has been shown that 1-deoxy-D-xylulose (DX) can be incorporated into carotenoids, plastoquinone and the phytol moiety of chlorophylls (Arigoni *et al.*, 1997; Lichtenthaler *et al.*, 1997; Schwender *et al.*, 1997), probably after conversion to DX5P. In addition, cDNAs encoding DXS have been cloned from several plant species, including peppermint (Lange *et al.*, 1998) and pepper (Bouvier *et al.*, 1998). After the identification of the *Escherichia coli dxs* gene sequence, it was found that the gene disrupted in the previously reported *Arabidopsis thaliana CLA1* mutant (Mandel *et al.*, 1996) encoded DXS (Lois *et al.*, 1998). *CLA1* mutant plants show a very early arrest of chloroplast development, lack of chlorophyll and carotenoid pigments, and an albino phenotype (Mandel *et al.*, 1996). Therefore DXS appears to be required for the biosynthesis of isoprenoids essential for plastid function. DX5P is a biosynthetic intermediate not only for IPP synthesis, but also for thiamine and pyridoxol in plastids (Julliard, 1992; Julliard and Douce, 1991). Thus it has been proposed that the first regulatory step in IPP and isoprenoid formation might be the synthesis of ME4P from DX5P (Lange and Croteau, 1999; Takahashi *et al.*, 1998). However, this possibility is yet to be tested.

Because of its novelty, the contribution of the Rohmer pathway to the control of plastid isoprenoid biosynthesis has not yet been evaluated. Fruit development in tomato constitutes a good model system to investigate the regulation of plastid isoprenoid biosynthesis. Young developing fruits contain chloroplasts that synthesize isoprenoids not only for photosynthesis-related processes, but also for gibberellin and abscisic acid biosynthesis (Gillaspy *et al.*, 1993). During ripening, however, chloroplasts differentiate into chromoplasts in a process that involves degradation of chlorophylls and a massive accumulation of carotenoids (particularly lycopene), causing the fruit colour to change from green to red (Bartley and Scolnik, 1995; Gillaspy *et al.*, 1993; Khudairi, 1972). In order to characterize the role of the first enzyme of the Rohmer pathway in plastid isoprenoid biosynthesis, we have cloned a cDNA corresponding to the single tomato *DXS* gene and demonstrated that it encodes a functional DXS protein which is targeted to plastids *in vivo*. Analysis of tomato *DXS* expression indicated developmental and organ-specific regulation of mRNA levels, and a strong correlation with carotenoid accumulation in tomato fruit.

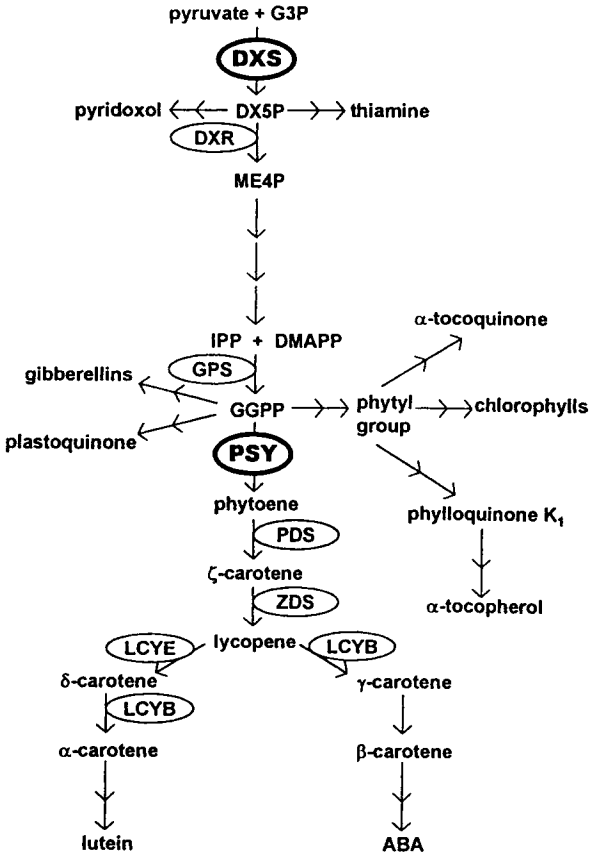

Figure 1. Plastid isoprenoid biosynthesis pathway.
G3P, D-glyceraldehyde 3-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DX5P, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; ME4P, 2-*C*-methyl-D-erythritol 4-phosphate; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; GPS, geranylgeranyl pyrophosphate synthase; GGPP, geranylgeranyl pyrophosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; ABA, abscisic acid.

In addition, our data indicate that DXS is a potentially key regulatory enzyme for carotenoid biosynthesis.

Results

Cloning of a tomato cDNA encoding DXS

An *Arabidopsis* 1.5 kb cDNA fragment corresponding to the *CLA1* gene (Mandel *et al.*, 1996) was used as a probe to screen a tomato leaf cDNA library. Two positive clones were isolated which, after DNA sequencing, were shown to correspond to overlapping cDNAs. The longer, LeDXS1, contains a 2568 bp insert with a 2160 bp open reading frame flanked by a 156 bp 5′ UTR and a 252 bp 3′ UTR. The protein encoded by this cDNA has 719 amino-acid residues and a predicted molecular mass of 77.6 kDa (Figure 2). The predicted polypeptide shows high similarity to published DXS proteins from pepper (96% identity), *Arabidopsis*

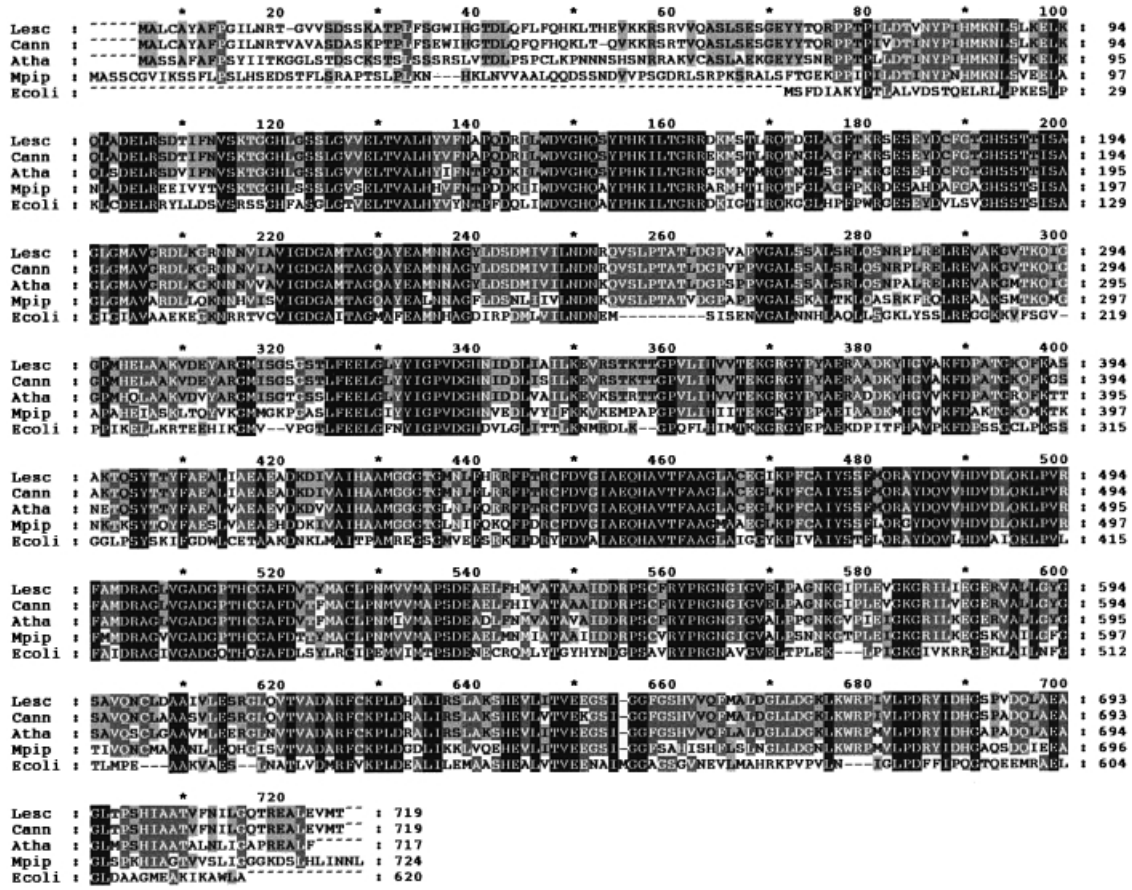

Figure 2. Multiple alignment of DXS amino acid sequences.
Sequences from the following organisms are included: tomato (*Lycopersicon esculentum*; AF143812); pepper (*Capsicum annuum* CapTKT2, Bouvier *et al.*, 1998); *Arabidopsis thaliana* (CLA1, Mandel *et al.*, 1996); peppermint (*Mentha* × *piperita*, Lange *et al.*, 1998); and *Escherichia coli* (Lois *et al.*, 1998). Identical residues are shown as white inside black boxes (when they are conserved in the five sequences); white inside grey boxes (conserved in four of the sequences); and black inside grey boxes (conserved in three sequences).

(83%) and peppermint (64%), and it is also similar to bacterial DXS (i.e. 60% similarity with the *E. coli* enzyme). Plant DXS proteins contain an N-terminal domain which is not present in the microbial enzyme (Figure 2). This domain is poorly conserved but shows the general features of plastidial targeting sequences, including an abundance of the hydroxylated residues serine and threonine and a shortage of the acidic residues aspartic acid and glutamic acid (Von Heijne *et al.*, 1991). Genomic DNA blot hybridization experiments, using as a probe the complete *LeDXS1* cDNA sequence, indicated the presence of a single copy gene in tomato (data not shown).

To confirm the biochemical function of the protein encoded by the cloned tomato cDNA, a complementation assay was carried out using the *E. coli* strain MC4100 *dxs::CAT*, in which the *dxs* gene had been disrupted by insertion of the *CAT* gene, encoding chloramphenicol acetyltransferase (Charon *et al.*, 2000). The disruption of the *dxs* gene is lethal due to the block in the biosynthesis of essential DX5P-derived compounds (isoprenoids, thiamin and pyridoxol). However, the mutant cells can grow in media supplemented with DX (Charon *et al.*, 2000). For the complementation assay, the protein encoded by the tomato *LeDXS1* cDNA was expressed in *E. coli dxs::CAT* under the control of the P_{BAD} promoter, which can be activated with arabinose and repressed with

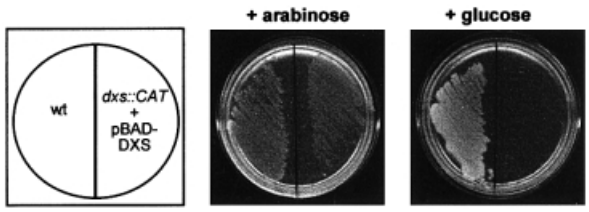

Figure 3. Complementation assay of *E. coli dxs* mutant.
E. coli MC4100 *dxs::CAT* cells transformed with plasmid pBAD-DXS, in which the expression of the protein encoded by the tomato *LeDXS1* cDNA is under the control of the P_{BAD} promoter, were grown on 2 × TY plates supplemented with either 0.02% arabinose or 0.2% glucose (right side of plates). Wild-type *E. coli* strain MC4100 was grown on the same plates as a control (left side).

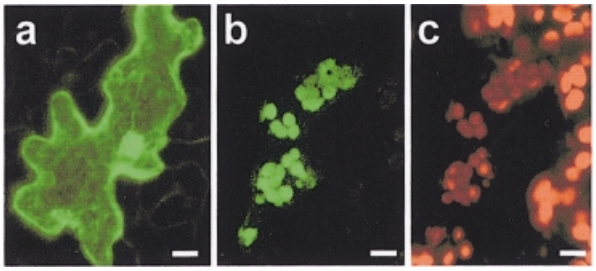

Figure 4. Subcellular localization of DXS-GFP in tomato.
Leaves were microbombarded with constructs to express either GFP (a) or the fusion protein DXS-GFP (b, c). Green fluorescence from the GFP proteins (a, b) and red fluorescence from chlorophyll (c) was detected by confocal microscopy. Bars, 10 µm.

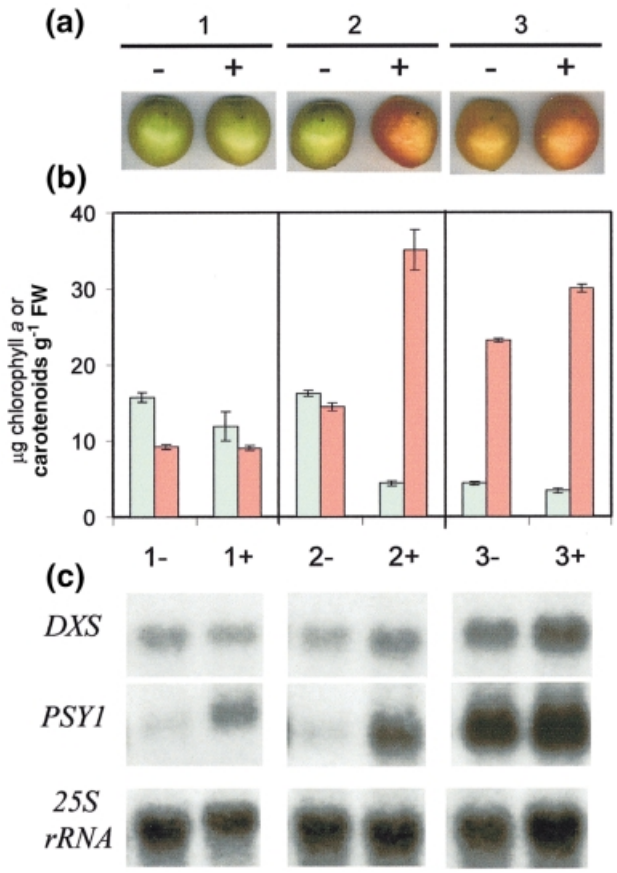

Figure 6. Effect of DX injection on tomato fruit development.
Mature green fruits were longitudinally cut in two halves and either a DX (+) or a control (–) solution were injected into each half. After 3 days, fruits were classified into groups 1, 2, or 3 according to the visible accumulation of carotenoid pigments.
(a) Representative fruits of each group.
(b) Measurement of chlorophyll *a* (green) and total carotenoids (orange) in the fruit halves shown in (a). Columns represent means and bars represent standard errors of three replicas.
(c) Blot with RNA samples from the fruit halves shown in (a) hybridized with *DXS*, *PSY1* and *25S rRNA* probes.

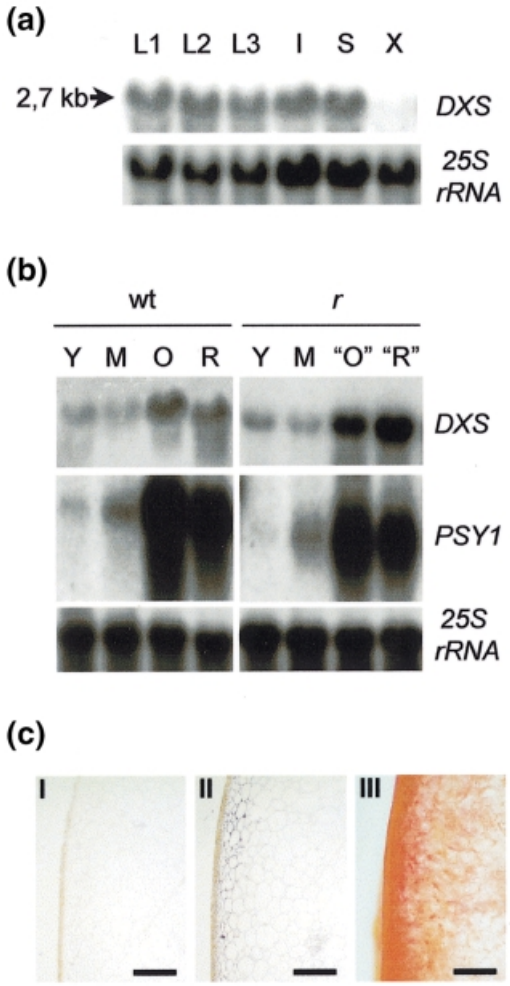

Figure 5. Analysis of tomato *DXS* expression pattern.
(a) Blot with RNA samples from tomato leaves (L1, young; L2, expanding; L3, fully expanded); inflorescences (I); stems (S); and roots (X) hybridized with a tomato *DXS* probe. The size of the transcript detected is shown. Exposure was carried out for 4 days.
(b) Blots with RNA samples from tomato Ailsa Craig wild-type young (Y); mature green (M); orange (O); and red ripe (R) fruit; and mutant *yellow flesh* (*r*) young (Y); mature green (M); orange-like (O); and yellow ripe (R) fruit were hybridized with probes for *DXS* and *PSY1*, and exposed for 2 days. The blots described in (a) and (b) were also hybridized with a *25S rRNA* probe to compare the RNA amounts loaded in each lane.
(c) Localization of *DXS* mRNA and carotenoid pigments on tomato fruit sections. I, *In situ* hybridization of an orange fruit section (8 µm) with a tomato *DXS* sense riboprobe; II, *in situ* hybridization of a similar section (8 µm) with a tomato *DXS* antisense riboprobe; III, red fruit hand-made section. Bars, 0.5 mm.

glucose. Induction with arabinose overcame DX auxotrophy of the transformed *dxs::CAT* mutant cells (Figure 3). However, repression with glucose prevented growth of the transformed mutant cells. These results demonstrate that the cloned tomato cDNA encoded a functional DXS.

Tomato DXS is targeted to plastids

The N-terminal region of plant DXS has been suggested to be a plastid targeting sequence (Lange *et al.*, 1998; Mandel *et al.*, 1996). However, the only reported experimental evidence on the plastidial localization of this enzyme is

based on the cross-reaction of plastid polypeptides of the predicted size with an anti-DXS antibody in pepper fruit (Bouvier *et al.*, 1998). To determine whether DXS is targeted to plastids *in vivo*, we fused the N-terminal 430 residues of tomato DXS to sGFP, a soluble codon-optimized green fluorescent protein (Rodríguez-Concepción *et al.*, 1999), for expression of the corresponding fusion protein (DXS-GFP) in living cells. Tomato leaves were microbombarded with constructs to transiently express either GFP or DXS-GFP under the control of the CaMV 35S promoter. As expected, green fluorescence corresponding to GFP was localized in the cytoplasm and the nucleus (Figure 4a). Fluorescence from DXS-GFP, however, colocalized with chlorophyll autofluorescence (Figure 4b,c). This result confirms that tomato DXS is targeted to chloroplasts *in vivo*, in agreement with the proposed role of this enzyme in the biosynthesis of plastidic isoprenoids.

Organ-specific and developmental expression of tomato DXS

To gain more insights into the function of DXS in tomato plants, the expression pattern of the *DXS* gene was analysed in several tissues by RNA blot hybridization. *DXS* transcripts are abundant in young, developing, and fully expanded leaves, inflorescences, and stems (Figure 5a). *DXS* transcripts in roots, however, were hardly detectable, even after long exposure. The level of *DXS* transcripts in young and mature green fruit was similar to that in the other photosynthetically active shoot tissues, although a slight decrease in *DXS* mRNA accumulation was detected in mature green fruit as compared to young fruit (Figure 5b). However, the level of *DXS* transcripts increased greatly during fruit ripening. The highest level of transcripts was detected in orange fruit, and decreased during the latest stages of ripening (Figure 5b). These results show that *DXS* mRNA accumulation in tomato is controlled by organ-specific and developmental signals. They also show that the increase in *DXS* mRNA accumulation correlates with the transition from mature green to orange fruit, suggesting that *DXS* induction is associated with the activation of carotenoid biosynthesis at the onset of ripening. Therefore it could be expected that cells actively synthesizing carotenoids would have the highest level of *DXS* expression. To study this possibility, *in situ* hybridization experiments were carried out on tomato orange fruit sections. *DXS* transcripts were predominantly localized in the outer layers of cells of the fruit pericarp (Figure 5c). Hand-made sections of orange and red fruit pericarps also showed that most of the orange and red carotenoid pigments were distributed in the same peripheral area of the pericarp (Figure 5c). These results further support a role for DXS in carotenoid biosynthesis during tomato fruit ripening.

Fruit DXS *mRNA accumulation pattern is affected by PSY1 activity*

It has been proposed that PSY1, the fruit-specific isoform of phytoene synthase (which catalyses the formation of phytoene from two molecules of GGPP; Figure 1), is the limiting enzyme for carotenoid biosynthesis during tomato fruit ripening (Bartley and Scolnik, 1995). To compare the expression patterns of *PSY1* and *DXS* genes during fruit development, the same filter used with the *DXS* probe (Figure 5b) was stripped and rehybridized with a *PSY1* probe. Similarly to *DXS*, a significant accumulation of *PSY1* mRNA was detected in ripening fruit, reaching the highest levels at the orange stage and slightly decreasing in red, ripe fruit (Figure 5b). The similar pattern of expression of *DXS* and *PSY1* suggests a coordinated role during carotenoid biosynthesis in ripening fruit. However, the kinetics of *DXS* and *PSY1* transcript accumulation were clearly different during the transition from growing to ripening fruit. Consistent with previous results (Giuliano *et al.*, 1993; Ronen *et al.*, 1999), the *PSY1* mRNA level began to increase in mature green fruits before the first colour changes were seen (Figure 5b). The observation that the induction of *PSY1* transcript accumulation precedes that of *DXS* suggests that changes in the level of carotenoid intermediates and/or end-products associated with higher PSY1 activity in mature green fruit might be involved in the regulation of *DXS* expression during fruit ripening. This possibility was tested by analysing *DXS* expression in the *yellow flesh* (*r*) mutant. The *r* mutation is known to affect the *PSY1* gene, resulting in the expression of a shorter mRNA that encodes a non-functional enzyme (Fray and Grierson, 1993). Therefore, the *r* fruit contains virtually no carotenoids, although it shows a yellow colour that is primarily due to the accumulation of the flavonoid chalconaringenin.

The results of the RNA blot analysis of *DXS* expression in fruits from the *r* mutant are shown in Figure 5(b). *DXS* transcript levels in young and mature green fruits of the *r* mutant were similar to those in wild-type fruits. Fruit ripening stages in the *r* mutant were defined as orange-like (when chlorophyll degradation and pigment accumulation changed fruit colour from green to yellow) and yellow ripe (yellow soft fruit). Although this colour- and softening-based staging system for ripening may not be accurate, the pattern of accumulation of *PSY1* transcripts in wild-type fruits was identical to that of the shorter *PSY1* mRNAs in the selected *r* fruits (Figure 5b; Ronen *et al.*, 1999), suggesting that the fruits from *r* and wild-type tomato plants used for the experiment were at similar ripening stages. The onset of ripening in *r* orange-like fruits was also associated with a high increase in *DXS* mRNA accumulation. However, *DXS* transcript level did not decrease in later stages of ripening, leading to mRNA

levels that were much higher than those detected in ripe wild-type fruits (Figure 5b). This result shows that PSY1 activity and carotenoid synthesis are not required for the induction of *DXS* transcript accumulation at the beginning of ripening, but they seem to be involved in the down-regulation of *DXS* expression in the last stages of ripening.

DX induces expression of PSY1 *and* DXS, *and carotenoid accumulation*

The strong correlation between the temporal and spatial patterns of *DXS* expression and carotenoid accumulation suggested that DXS activity might be limiting for carotenoid biosynthesis during tomato fruit ripening. To mimic the effect of increased DXS activity on carotenoid accumulation, DX was directly injected into mature green tomato fruit. DX was used instead of DX5P to overcome problems associated with uptake of the phosphorylated compound into cells. Previous reports have shown that DX can readily enter the plastid isoprenoid pathway when externally supplied to plant cells (Arigoni *et al.*, 1997; Lichtenthaler *et al.*, 1997; Schwender *et al.*, 1997).

A total of 14 mature green fruits were used in three independent experiments. Each fruit was longitudinally cut in two halves. A syringe with a fine needle was used to inject 50 µl of a 100 mM DX solution to one of the halves. The same volume of a control solution without DX was injected to the other half. After 3 days at room temperature, the fruits were classified into three different groups according to the colour of the corresponding halves (Figure 6). In two out of the 14 fruits (group 1), both halves remained green. In contrast, in seven fruits the DX-injected half of each fruit became red, whereas the control half remained green (group 2). In the remaining five fruits, carotenoid pigments accumulated in both halves, but the one treated with DX typically showed a stronger red colour (group 3).

Representative fruits from the three groups were selected (Figure 6a), and their corresponding halves were frozen and ground in liquid nitrogen to a fine powder which was used for measurement of both chlorophyll and carotenoid contents (Figure 6b) and RNA analysis (Figure 6c). Although no visible colour difference was observed between the two halves of group 1 fruit, chlorophyll *a* content in the DX-treated half was lower than in the control half, whereas the carotenoid level was similar (Figure 6b). The DX-treated half of group 1 fruit showed a higher level of *PSY1* mRNA, although the *DXS* transcript level remained unchanged (Figure 6c). In the fruits from groups 2 and 3, DX injection induced carotenoid accumulation (Figure 6b). In group 2 fruit, the DX-injected half showed significantly less chlorophyll *a* and more carotenoids than the control half. In these fruits, DX induced not only *PSY1* but also *DXS* gene expression (Figure 6c). The control half from group 3 fruit had low chlorophyll *a* and high carotenoid contents (Figure 6b), and it showed higher levels of *DXS* and *PSY1* transcripts than those of control fruits from groups 1 and 2 (Figure 6c). The DX-injected half of group 3 fruit, however, again accumulated more carotenoids and showed higher levels of *DXS* and *PSY1* transcripts than the control. The different level of chlorophyll *a*, carotenoids, and *DXS* and *PSY1* transcripts in the control halves from group 1, 2 and 3 fruits shows that their starting metabolic status was different. This was expected as 'mature green' is a relatively broad developmental definition that can span a few days. However, our experimental design suggests that DX injection activates accumulation of *PSY1* transcripts and eventually carotenoids independently of the fruit stage. Taken together, our data suggest that synthesis of DX5P by DXS is probably a key regulatory step for carotenoid biosynthesis.

Discussion

Since the discovery of the mevalonate-independent pathway for the synthesis of plastid isoprenoids (Rohmer pathway), the full elucidation of the pathway and its regulatory role in the synthesis of specific plastidic isoprenoid end-products represents a novel major challenge in plant cell metabolism. Here we present the molecular characterization of tomato DXS, the first enzyme of the Rohmer pathway, and propose its regulatory role in the biosynthesis of carotenoids during tomato fruit ripening.

Using *in vivo* systems, we have demonstrated that the protein encoded by the cloned tomato LeDXS1 cDNA is a functional DXS that is targeted to chloroplasts, where it probably synthesizes DX5P for plastid isoprenoid biosynthesis. In agreement, analysis of the *DXS* expression pattern showed a clear correlation between *DXS* mRNA accumulation and plastid isoprenoid requirements of the tissue. The level of *DXS* mRNA was similar in all the photosynthetic tissues tested, whereas it was very low in roots (Figure 5). Most of the plastid isoprenoid products are related to photosynthesis (Figure 1), and therefore a more active *DXS* expression in shoot tissues compared to the root can be expected. Consistently, the *CLA1* gene encoding *Arabidopsis DXS* is positively regulated by light (Mandel *et al.*, 1996). Nevertheless, very low levels of expression of *DXS* in tomato roots and in dark-grown *Arabidopsis* seedlings can be detected, suggesting that DXS activity is required, at least at a basal level, in all cell types.

DXS expression also correlates with the production of specific isoprenoids in other plants. Peppermint leaves contain oil gland secretory cells highly specialized for isoprenoid (monoterpene) accumulation. In these cells, *DXS* mRNA level is highest during the first stages of leaf

development, preceding the peak of monoterpene biosynthesis (Lange *et al.*, 1998). In tomato, *DXS* expression in young to fully expanded leaves remained constant, consistent with the fact that no specific isoprenoid product is accumulated at high levels during leaf development. Unlike leaves, however, tomato fruit showed a developmentally regulated pattern of *DXS* expression. Young and mature green fruit had *DXS* transcript levels similar to those detected in other photosynthetic tissues, although a slight decrease in *DXS* transcript level was observed when young fruit developed into mature green fruit. This *DXS* down-regulation correlated with a lower accumulation of photosynthesis-related isoprenoids such as carotenoids and chlorophylls (Fraser *et al.*, 1994; Giuliano *et al.*, 1993), and with a decreased requirement for other plastid isoprenoid-derived products such as gibberellins and abscisic acid in mature green fruit (Gillaspy *et al.*, 1993). In contrast, a strong induction of *DXS* expression was observed during fruit ripening at the stages of high rate of carotenoid accumulation, although the *DXS* transcript level decreased in ripe fruit (Figure 5). *DXS* expression is also up-regulated in correlation with carotenoid accumulation in pepper fruit (Bouvier *et al.*, 1998). Furthermore, the accumulation of *DXS* transcripts in cells from the outer pericarp tissue of tomato orange fruit closely paralleled the localization of carotenoid pigments. In summary, the pattern of *DXS* expression was almost identical to that reported for the accumulation of carotenoids throughout fruit development (Fraser *et al.*, 1994; Giuliano *et al.*, 1993), except in the last stages of ripening. Carotenoid biosynthesis is a highly controlled process which differs in chloroplast- and chromoplast-containing tissues, suggesting a specific regulation of genes and enzymes of the carotenoid pathway at each plastid developmental stage (Fraser *et al.*, 1994; Giuliano *et al.*, 1993). In the transition from green to orange fruit, the carotenoid profile changes dramatically. In green fruits, the main carotenoid products are lutein and β-carotene (Fraser *et al.*, 1994; Ronen *et al.*, 1999). During ripening, however, both lycopene β- and ε-cyclase activities (LCYB and LCYE, respectively; Figure 1) decrease to undetectable levels and lycopene accumulates in chromoplasts, resulting in the characteristic red colour of tomato ripe fruit (Fraser *et al.*, 1994; Ronen *et al.*, 1999). Our expression studies in the *r* mutant suggest that the qualitative and/or quantitative changes in ripe fruit carotenoids contribute to *DXS* down-regulation (Figure 5). The fact that *DXS* mRNA levels do not decrease (but increase) as a result of the accumulation of higher levels of orange-red carotenoids after injection of DX to mature green fruit (Figure 6) confirm that the carotenoid-induced *DXS* down-regulation is specific to the last stages of ripening. End-product regulation of other tomato genes encoding enzymes of the carotenoid pathway, such as phytoene desaturase (PDS; Figure 1), has also been reported (Corona *et al.*, 1996). The complex regulation of tomato *DXS* gene expression is consistent with the current model of control of carotenoid biosynthesis by modulation of the transcriptional activities of genes encoding enzymes of the pathway (Corona *et al.*, 1996; Fraser *et al.*, 1994; Ronen *et al.*, 1999).

The pattern of *DXS* mRNA accumulation is similar to the pattern of expression of *PSY1*, the gene reported to encode the committed enzyme in carotenoid biosynthesis during tomato fruit development (Bartley and Scolnik, 1995). But unlike *DXS*, *PSY1* expression (Figure 5b) and activity (Fraser *et al.*, 1994) are induced before the onset of ripening, in the mature green stage. Induction of PSY1 activity, however, does not result in increased carotenoid levels (which actually decrease at this stage of development; Fraser *et al.*, 1994; Giuliano *et al.*, 1993), as would be expected from the current view of PSY1 as the regulatory enzyme in fruit carotenoid biosynthesis (Bartley and Scolnik, 1995). The activation of *PSY1* expression preceding the increase in *DXS* transcript levels was also observed after DX injection (Figure 6). This induction of *PSY1* mRNA accumulation by DX feeding and the modulation of *DXS* expression by PSY1 activity during the last stages of fruit development (Figure 5b) suggest a significant cross-talk between PSY1 and DXS activities that may contribute to the fine regulation of carotenoid accumulation in tomato fruit. Assuming that the changes in *DXS* mRNA levels result in similar changes in DXS enzyme activity, we propose an alternative model for the regulation of carotenoid synthesis during tomato fruit development (Figure 7). In this model, DXS rather than PSY1 would control the start of massive carotenoid accumulation when tomato fruit enters the ripening phase. It is possible that PSY1 activity precedes the increase in DX5P synthesis, so the bulk of GGPP derived from DX5P in the first stages of ripening may be immediately channelled to phytoene and readily enter the carotenoid biosynthesis pathway. Thus PSY1 activity would be limiting for tomato fruit carotenoid biosynthesis only if non-limiting levels of the GGPP substrate were available.

Since DX5P is a precursor not only of isoprenoids, but also of thiamine and pyridoxol (Julliard, 1992; Julliard and Douce, 1991), it has been suggested that the conversion of DX5P into ME4P catalysed by DX5P reductoisomerase (DXR; Figure 1) could be the first regulatory step in the Rohmer pathway (Lange and Croteau, 1999; Takahashi *et al.*, 1998). However, the temporal and spatial expression patterns of the tomato *DXR* gene during fruit development do not support the hypothesis that the reaction catalysed by DXR could be such a regulatory step (M.R.C., L.M.L. and A.B., unpublished results). Our model (Figure 7) proposes that DXS activity (and therefore DX5P availability) is limiting for carotenoid synthesis during the first stages of ripening. In agreement, injection experiments (Figure 6)

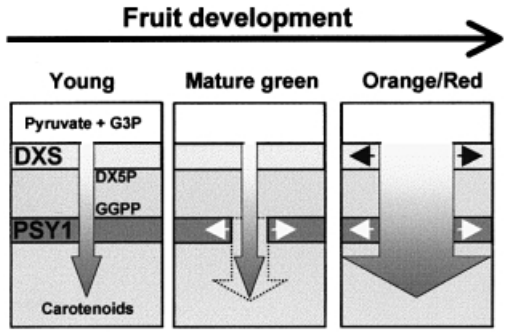

Figure 7. Proposed model of the role of DXS and PSY1 in the regulation of carotenoid biosynthesis in tomato fruit.
The model is based on the expression pattern of *DXS* and *PSY1* during fruit development and the DX injection experiments in mature green fruit. DXS and PSY1 activities are represented as gates that control flux (vertical arrows) from substrates to products. In young fruit, the coordinated activities of the two enzymes result in the production of carotenoids for photosynthesis-related processes. In mature green fruit, *DXS* transcript level does not significantly change, but there is an increase in *PSY1* expression and activity. Despite the proposed regulatory role of PSY1 for carotenoid biosynthesis, the higher level of PSY1 activity in mature green fruit does not result in more carotenoids, probably because DXS activity does not provide enough DX5P to be channelled to GGPP, the substrate of PSY1. In orange and red fruit, the high increase in both *DXS* and *PSY1* expression leads to massive accumulation of carotenoids.

showed that an increase in the levels of DX (or most probably its metabolically active form, DX5P, which mimics a specific increase in DXS activity) results in up-regulation of the expression of *PSY1* and eventually of *DXS*, and increased carotenoid content in the fruit. These observations indicate that DXS activity is indeed the first potentially regulatory step in the biosynthesis of fruit carotenoids, at least during early stages of ripening. Furthermore, in addition to induction of *PSY1* expression, DX injection activated other processes typically associated with the onset of ripening such as chlorophyll *a* degradation (Figure 6) and down-regulation of the *rbcS2* gene (M.R.C., L.M.L. and A.B., unpublished results), both of which precede carotenoid accumulation (Fraser *et al.*, 1994; Gillaspy *et al.*, 1993; Khudairi, 1972). We speculate that increased synthesis of DX5P might contribute to the induction of the developmental process of fruit ripening as a whole. Since ripening is an aspect of development that is unique to fruit, it is possible that the proposed regulatory character of DXS is fruit-specific. However, overexpression of *DXS* in *E. coli* cells carrying a plasmid harbouring lycopene biosynthesis genes leads to higher accumulation of lycopene and ubiquinone (Harker and Bramley, 1999), suggesting that DXS activity may also be limiting for isoprenoid biosynthesis in microorganisms.

In conclusion, our results support previous studies showing that carotenogenesis in tomato fruit is independent of the mevalonate pathway (Rodríguez-Concepción and Gruissem, 1999), and provide further evidence that the isoprenoid intermediates for carotenoid biosynthesis derive from the Rohmer pathway. In addition, we show several lines of experimental evidence that synthesis of DX5P is a key rate-limiting step for carotenoid biosynthesis in tomato fruit. Our results change the current view that PSY1 is the only regulatory enzyme in tomato fruit carotenogenesis, and point towards a coordinated role of both DXS and PSY1 in the control of carotenoid synthesis during ripening. This opens up a new perspective to the study and eventual manipulation of carotenoid accumulation in tomato fruit.

Experimental procedures

Plant material and treatments

Seeds from tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) wild type and *yellow flesh* mutant (*r*) were a gift from the laboratories of Dr P.M. Bramley and Dr W. Gruissem. Plants were grown under controlled greenhouse conditions. Green fruit development stages were defined as described (Gillaspy *et al.*, 1993) and are referred to as young (up to 1.5 cm in diameter) and mature green. Orange and ripe fruit stages in the wild type were also defined as described (Gillaspy *et al.*, 1993). Ripening stages in the *r* mutant were defined as orange-like (when chlorophyll degradation and pigment accumulation changed fruit colour from green to yellow), and yellow ripe (yellow soft fruit). 1-Deoxy-D-xylulose (DX) was prepared basically as described (Lois *et al.*, 1998). For injection experiments, a total of 14 mature green fruits were collected from the plant and longitudinally cut in two halves. Immediately, the open sections of both halves were protected from desiccation with Saran Wrap. A 0.5 ml syringe with a fine needle was used to inject 50 µl of 100 mM DX in 0.1% (v/v) Tween 20 through the pericarp to one of the halves. The same volume of 0.1% (v/v) Tween 20 was injected into the other half as a control. After 3 days at room temperature, both halves were photographed, frozen in liquid nitrogen, and stored at –80°C until used for experiments.

cDNA cloning and analysis

Plasmid pH 2A12, containing a 2.6 kb cDNA which encodes the full-length CLA1 (DXS) protein from *Arabidopsis thaliana*, was obtained from the DNA Stock Center at ABRC, Ohio State University (EST 22939 CD4-16, GenBank accession number W43562). A 1.5 kb *Xba*I-*Eco*RI fragment from pH 2A12 was used as a probe to screen a tomato leaf cDNA library (kindly provided by Dr Salomé Prat). After screening 90 000 λgt11 plaques under low-stringency conditions (Sambrook *et al.*, 1989), two positive clones (LeDXS1 and LeDXS2) were identified and used for further analysis. The cloned cDNAs were excised from the λ vector by digestion with *Bam*HI and the fragments released were cloned into pBluescript (Stratagene). Both strands were sequenced with the ABI PRISM DNA Sequencing kit (Amersham) using vector primers and specific primers. Sequencing showed that both clones corresponded to overlapping cDNAs. The plasmid containing the longer clone, LeDXS1 (encoding a full-length protein with homology to DXS), was named pLeDXS. Sequence analyses were performed using the GCG 9.0 software package (Genetics Computer Group Inc.) and the NCBI BLAST program available on

the web (www.ncbi.nlm.nih.gov/). Multiple alignment was performed using the PILEUP program (GCG) with the default options.

E. coli *complementation*

An *Nde*I restriction site was introduced at the ATG translation start codon of the tomato cDNA in plasmid pLeDXS with the QuickChange site-directed mutagenesis kit (Stratagene) using Pfu DNA polymerase (Gibco-BRL) and the complementary primers PTom-Nde (5′-CAGTTGAATTGACTACATATGGCTTTGTGTGC-3′) and PTom-Nde-C (5′-GCACACAAAGCCATATGTAGTCAATTCAACTG-3′), as specified by the supplier. The resulting plasmid was designated pLeDXSmut. A modified version of plasmid pBAD-GFPuv (Clontech) was created by removing the *Nde*I site located at position 4926 by site-directed mutagenesis with the oligonucleotide PBAD-mut1 (5′-CTGAGAGTGCACCATCTGCGGTGTGAAATACC-3′) as mutagenic primer. The resulting plasmid was designated pBAD-M1. Expression plasmid pBAD-DXS was constructed by cloning the 2.4 kb *Nde*I-*Kpn*I fragment generated by partial digestion of pLeDXSmut with *Nde*I into the corresponding sites of plasmid pBAD-M1. Plasmid pBAD-DXS was used to transform *E. coli* MC4100 *dxs::CAT* cells (Charon *et al.*, 2000). One isolated colony was selected and streaked on 2 × TY plates supplemented with either 0.02% arabinose (to induce expression from the P_{BAD} promoter) or 0.2% glucose (to repress expression from the P_{BAD} promoter).

DNA and RNA analysis

Genomic DNA and RNA from several tomato tissues, except from fruit, were extracted according to Dean *et al.* (1985). Genomic DNA (10 μg) extracted from young leaves was digested with several restriction enzymes, size-fractioned by electrophoresis in a 0.8% (w/v) agarose gel, and blotted onto a Hybond-C nitrocellulose membrane (Amersham). Hybridization was carried out in 5 × SSC, 5 × Denhardt's, 1% (w/v) SDS and 500 μg ml^{-1} denatured salmon sperm DNA at 68°C for 18 h. The entire 2.6 kb tomato *DXS* cDNA excised from plasmid pLeDXS was ^{32}P-labelled with the Ready to Go kit (Pharmacia), and used as a probe. Washes were performed for 20 min at room temperature twice in 2 × SSC, 0.1% (w/v) SDS, twice in 0.2 × SSC, 0.1% (w/v) SDS, and at 42°C twice in 0.2 × SSC, 0.1% (w/v) SDS.

For fruit RNA extraction, fruits at different developmental stages were collected, seeds were removed, and the pericarp tissue was immediately frozen in liquid nitrogen. RNA was isolated as described (Rodríguez-Concepción and Gruissem, 1999). For RNA blot analysis, 10 μg from each sample were fractionated by gel electrophoresis in 1% (w/v) agarose gels containing 2% formaldehyde, and blotted onto Hybond-N+ membranes (Amersham). Probes were made by labelling cDNA inserts with the Ready to Go kit (Pharmacia). A *DXS* probe was made from a 726 bp *Nde*I-*Sac*II *DXS* cDNA fragment (corresponding to the predicted C-terminal region of the protein) excised from plasmid pLeDXS. The *PSY1* cDNA used as a probe contained the entire coding region and most of the 3′ UTR, and was isolated from tomato fruit RNA by RT–PCR with the primers PSY1F (5′-ACCATGGTTTTCTTGCTCAG-3′) and PSY1R (5′-GTCTAGAAGTCTCTCAAAGGAG-3′). The *25S rRNA* probe was made as described (Cunillera *et al.*, 1997). Hybridization was performed at 68°C in ExpressHyb7 hybrization solution (Clontech) for 1 h. Washes were carried out twice at room temperature in 1 × SSC, 0.1% (w/v) SDS and once at 45°C in 1 × SSC, 0.1% (w/v) SDS. Biomax (Kodak) film was used for exposures.

Microbombardment assay

A 1.4 kb *Kpn*I-*Nco*I fragment from the 5′ region of LeDXS1 cDNA was cloned into the same sites of a modified pGFP-MRC plasmid in which a *Kpn*I site had been created next to the *Xho*I site (Rodríguez-Concepción *et al.*, 1999). In the resulting construct, the CaMV 35S promoter directed the expression of the fusion protein DXS-GFP, in which the first 430 amino-acid residues of tomato DXS were fused in frame to the N-terminus of a synthetic green fluorescent protein (sGFP). Subcellular localization of the DXS-GFP fusion protein in tomato leaf epidermis cells was carried out by microbombardment with tungsten particles (1.0 μm) coated with the corresponding plasmid DNA, using a Biolistic PDS-1000/He system (Biorad) at a pressure of 1100 psi. After 24 h at 22°C with continuous light, samples were examined directly with a Leica TCS 4D Confocal Laser Scanning Microscope (CSLM). Green fluorescence corresponding to the GFP fusion proteins was detected using a BP515-525 filter after excitation with blue light at 488 nm. Red autofluorescence from chlorophyll was detected using a LP590 filter after excitation with green light at 568 nm.

In situ *hybridization*

Tomato orange fruit tissue was fixed in a solution containing 2% (v/v) formaldehyde, 4% (v/v) acetic acid and 60% (v/v) ethanol at 4°C for 2 days. After an ethanol series, dehydrated samples were embedded in paraffin (Paraplast Plus). Sections (8 μm) were used for *in situ* hybridization with digoxigenin-labelled (Roche) *DXS* RNA probes as previously described (Coen *et al.*, 1990). A 399 bp 3′ *Cla*I-*Bam*HI *LeDXS1* cDNA fragment cloned in pBluescript vector was used as a template to prepare the antisense probe with T3 RNA polymerase and 1 μg of plasmid DNA digested with *Cla*I. As a control, a sense probe was synthesized using T7 RNA polymerase and 1 μg of plasmid template linearized with *Xba*I. Specific activity of probes was checked by dot-blot analyses and working dilutions of 1 : 150 in 120 μl hybridization buffer were used. The hybridization signal was detected with NBT/BCIP (Roche) and the colorimetric reaction finally stopped with 10 mM Tris–HCl, 1 mM EDTA (pH 8) after 14 h incubation.

Chlorophyll and carotenoid analysis

Frozen powder (0.5 g) from the same fruit pericarp tissue used for RNA extraction was ground and mixed with 2 ml of methanol : -water (1 : 1) followed by addition of 4 ml chloroform. After 20 min, samples were centrifuged at 3000 r.p.m. for 10 min at room temperature. The lower phase was recovered and evaporated. Dried samples were resuspended in 100 μl ethylacetate, and quantification was performed in acetone as described (Lichtenthaler, 1987).

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