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Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors

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Summary

Carotenoids are isoprenoid pigments that function as photoprotectors, precursors of the hormone abscisic acid (ABA), colorants and nutraceuticals. A major problem for the metabolic engineering of high carotenoid levels in plants is the limited supply of their isoprenoid precursor geranylgeranyl diphosphate (GGPP), formed by condensation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) units usually synthesized by the methylerythritol phosphate (MEP) pathway in plastids. Our earlier work with three of the seven MEP pathway enzymes suggested that the first reaction of the pathway catalyzed by deoxyxylulose 5-phosphate synthase (DXS) is limiting for carotenoid biosynthesis during tomato (*Lycopersicon esculentum*) fruit ripening. Here we investigate the contribution of the enzyme hydroxymethylbutenyl diphoshate reductase (HDR), which simultaneously synthesizes IPP and DMAPP in the last step of the pathway. A strong upregulation of *HDR* gene expression was observed in correlation with carotenoid production during both tomato fruit ripening and *Arabidopsis thaliana* seedling deetiolation. Constitutive overexpression of the tomato cDNA encoding HDR in *Arabidopsis* did not increase carotenoid levels in etioplasts. By contrast, light-grown transgenic plants showed higher carotenoid levels and an enhanced seed dormancy phenotype suggestive of increased ABA levels. The analysis of double transgenic *Arabidopsis* plants overproducing both the enzyme taxadiene synthase (which catalyzes the production of the non-native isoprenoid taxadiene from GGPP) and either HDR or DXS showed a twofold stronger effect of HDR in increasing taxadiene levels. Together, the data support a major role for HDR in controlling the production of MEP-derived precursors for plastid isoprenoid biosynthesis.

Keywords: carotenoid, deetiolation, isoprenoid, MEP pathway, ripening, taxadiene.

Introduction

Carotenoids are an abundant group of isoprenoid pigments that are widely distributed in nature, with hundreds of structures known to date. They are synthesized by all photosynthetic organisms (plants, algae and cyanobacteria) and some non-photosynthetic bacteria and fungi. Animals, including humans, cannot synthesize carotenoids, although they are an essential source of retinoids and vitamin A. In plants, carotenoids are essential for protecting the photosynthetic apparatus from photooxidation and as precursors for the biosynthesis of abscisic acid (ABA). They also participate in light harvesting and furnish flowers and fruit with colors aimed at attracting animals that disperse pollen and seeds (Bartley and Scolnik, 1995; Demmig-Adams *et al.*, 1996; Fraser and Bramley, 2004; Hirschberg, 2001). A number of plant carotenoids and derived compounds (apocarotenoids) have an added industrial and nutritional value based on their colorant and aromatic properties, their activity as pro-vitamin A, and their ability to act as natural antioxidants that help to prevent some types of human cancer and degenerative diseases (Bartley and Scolnik, 1995;

Fraser and Bramley, 2004; Hirschberg, 2001; Sandmann, 2001). The recent discovery of such health-related properties and the increasing demand for natural products have spurred an unprecedented interest in the biotechnological overproduction of carotenoids in plants. The identification of the genes involved in carotenoid biosynthesis has opened the door to genetically manipulating this pathway in plants. Despite some successful approaches of metabolic engineering of carotenoids in crop plants, a major problem still to be solved is how to increase the flow of metabolic precursors toward carotenoid synthesis without affecting other interacting metabolic pathways (Fraser and Bramley, 2004; Hirschberg, 2001; Sandmann, 2001).

Structurally, carotenoids are tetraterpenes derived from the 5-carbon units isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), the universal precursors of all isoprenoid compounds (Croteau *et al.*, 2000; Cunningham and Gantt, 1998). Plants synthesize IPP and DMAPP by two independent pathways: the mevalonic acid (MVA) pathway, which produces cytosolic IPP, and the plastidial methylerythritol phosphate (MEP) pathway (Eisenreich *et al.*, 2001; Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002). In each compartment, condensation of IPP and DMAPP units leads to the synthesis of prenyl diphosphates of increasing size which serve as starting points for multiple branches leading to the final isoprenoid products. The addition of three molecules of IPP to one DMAPP unit catalyzed by the enzyme geranylgeranyl diphosphate (GGPP) synthase (GGDS) yields GGPP, the immediate precursor not only for carotenoids but also for the biosynthesis of gibberellins and the phytol tail of chlorophylls, phylloquinones and tocopherols (Figure 1). Although an exchange of prenyl diphosphates is known to take place between cell compartments (Eisenreich *et al.*, 2001; Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002), current evidence suggests that MVA-derived cytosolic precursors might contribute to the production of carotenoids only during specific developmental stages such as in etiolated seedlings (Rodríguez-Concepción *et al.*, 2004), whereas plant carotenoids synthesized in chloroplasts and chromoplasts derive mostly from the MEP pathway (revised in Lichtenthaler, 1999; Rodríguez-Concepción and Boronat, 2002).

While all the MEP pathway genes have been identified in bacteria and plants (reviewed in Rodríguez-Concepción and Boronat, 2002), much work is still ahead to analyze the contribution of the corresponding enzymes in the control of the flux of intermediates through the pathway and the supply of IPP and DMAPP for the synthesis of plastid isoprenoid end products such as carotenoids. Fruit development in tomato has been widely used as a system for these studies as a significant increase in the supply of isoprenoid precursors is required to support the massive accumulation of carotenoids that takes place during ripening (Gillaspy *et al.*, 1993; Lois *et al.*, 2000). Our previous work with three enzymes of the MEP pathway, deoxyxylulose 5-phosphate synthase (DXS), deoxyxylulose 5-phosphate reductoisomerase (DXR), and hydroxymethylbutenyl diphosphate (HMBPP) synthase (HDS, also called GCPE and ISPG) (Figure 1) established that only DXS appears to be limiting for carotenoid biosynthesis during tomato fruit ripening (Lois *et al.*, 2000; Rodríguez-Concepción *et al.*, 2001, 2003). The limiting nature of the reaction catalyzed by DXS for the biosynthesis of plastidial isoprenoids was also confirmed in photosynthetic tissues by constitutive overexpression of the corresponding gene in *Arabidopsis thaliana* plants (Estévez *et al.*, 2001). The contribution of other enzymes of the MEP pathway to regulate the supply of intermediates for plastid isoprenoid biosynthesis has not been established yet.

Before the MEP pathway was fully elucidated, genetic and biochemical approaches in *Escherichia coli* demonstrated that, unlike the MVA pathway, it branched at some point leading to the separate synthesis of IPP and DMAPP (Charon

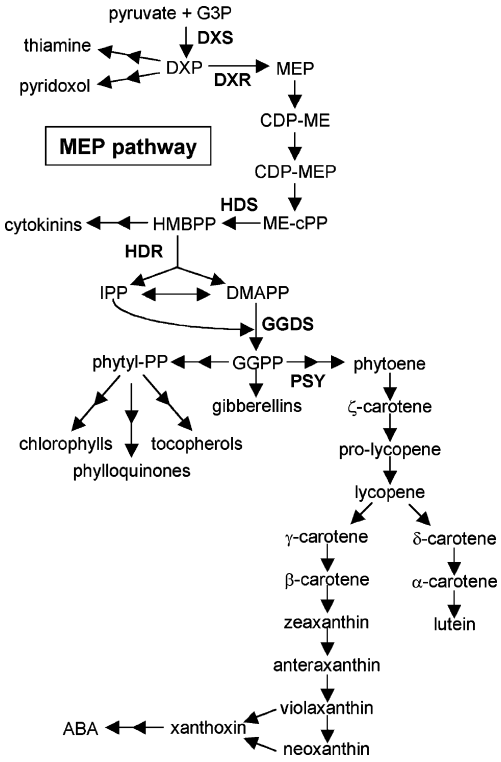

Figure 1. The pathway for carotenoid biosynthesis in plants. G3P, glyceraldehyde 3-phosphate; DXP, deoxyxylulose 5-phosphate; MEP, methylerythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-methylerythritol; CDP-MEP, 4-diphosphocytidyl-methylerythritol 2-phosphate; ME-cPP, methylerythritol 2,4-cyclodiphosphate; HBMPP, hydroxymethylbutenyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid. Some of the biosynthetic enzymes are indicated (in bold): DXS, DXP synthase; DXR, DXP reductoisomerase; HDS, HMBPP synthase; HDR, HMBPP reductase; GGDS, GGPP synthase; PSY, phytoene synthase.

et al., 2000; Rodríguez-Concepción *et al.*, 2000). Recent evidence has shown that the bacterial *lytB*/*ispH* gene product is responsible for such branching (Figure 1), directly converting HMBPP into a 5:1 mixture of IPP and DMAPP in the last step of the MEP pathway (Adam *et al.*, 2002; Altincicek *et al.*, 2002; Rohdich *et al.*, 2002; Wolff *et al.*, 2003). Overexpression studies using sequences from cyanobacteria (*Synechocystis*) and plants (*Adonis aestivalis*) showed that the activity of this enzyme, currently known as IPP/DMAPP synthase or HMBPP reductase (HDR, EC1.17.1.2), was limiting for isoprenoid biosynthesis in *E. coli* (Cunningham *et al.*, 2000). However, little more information is available on plant systems besides the results on the independent synthesis of IPP and DMAPP in plant plastids (Adam *et al.*, 2002; Hoeffler *et al.*, 2002), the reported abundance of HDR-encoding expressed sequence tags (ESTs) in the isoprenoid-rich glandular trichomes of mint leaves (Cunningham *et al.*, 2000), and the albino phenotype of plants with a silenced *HDR* gene (Gutierrez-Nava *et al.*, 2004; Page *et al.*, 2004). In this work we investigated the role of HDR in regulating the production of precursors for carotenoid biosynthesis.

Results

Identification of plant sequences encoding HDR homologues

To identify plant HDR sequences, the TBLASTN algorithm was used in a similarity search on the available EST databases with the *A. aestivalis* protein sequence (Cunningham *et al.*, 2000) as a query. In The Institute for Genomic Research (TIGR) database, the search retrieved several polypeptides deduced from ESTs in several other plants, including rice (TC200700), *Arabidopsis* (TC183666), potato (TC57777), tomato (TC124188), lettuce (TC12240), and grape (TC14300). The EST sequence data are consistent with a single gene encoding HDR in plants. Figure 2 shows an

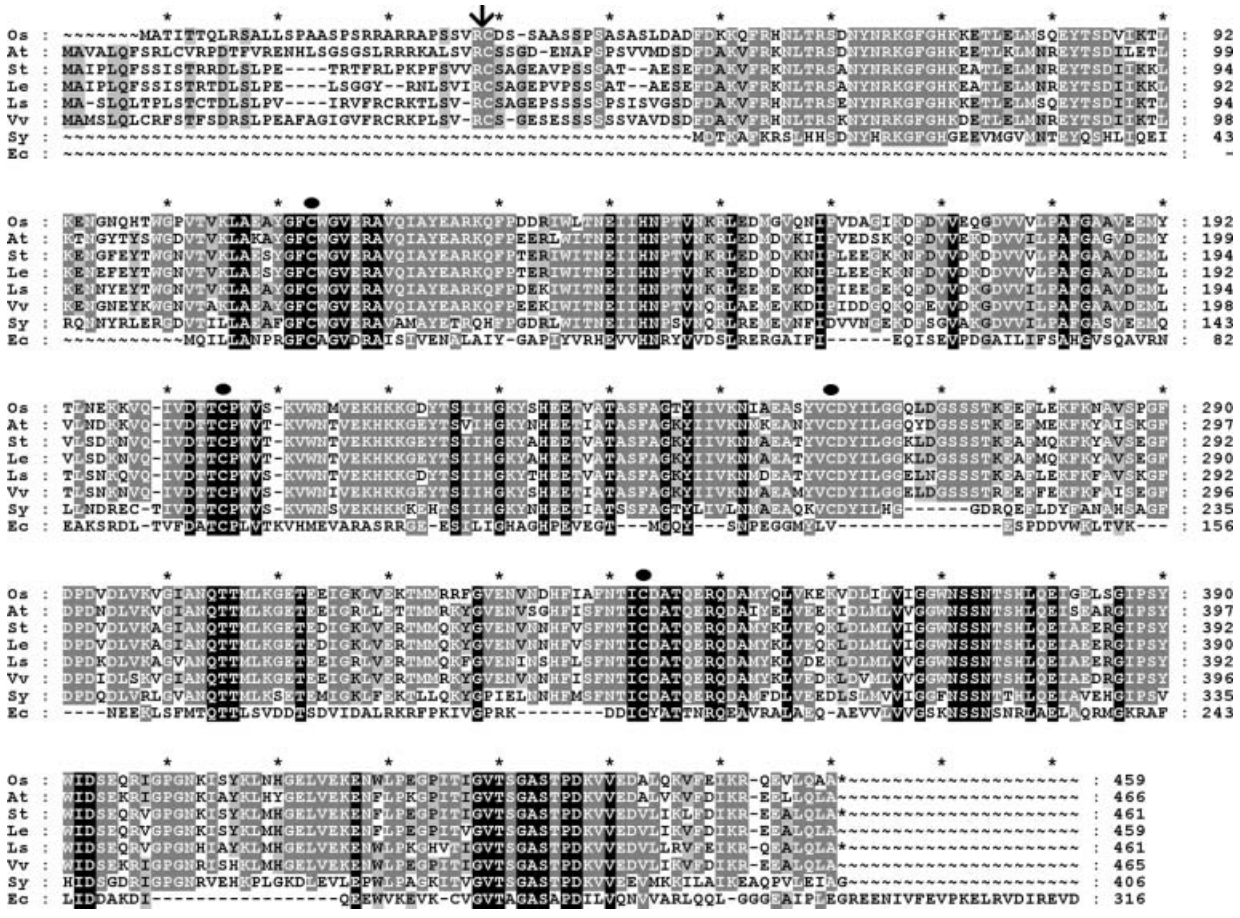

Figure 2. Multiple alignment of HDR sequences. Rice (Os, TC200700), *Arabidopsis* (At, TC183666), potato (St, TC57777), tomato (Le, TC124188), lettuce (Ls, TC12240), grape (Vv, TC14300), *Synechocystis* (Sy, Q55643), and *Escherichia coli* (Ec, P22565) sequences are deposited in the TIGR and Swiss-Prot databases. Numbers indicate the position of amino acid residues. Conserved residues are highlighted (in black when present in all the sequences). Arrow indicates the position predicted with the ChloroP algorithm for the cleavage of the plastid-targeting peptides from the plant proteins. Dots mark the position of conserved cysteine residues.

alignment of the deduced plant HDR sequences with those encoded by the *lytB/ispH* gene from *Synechocystis* (Q55643) and *E. coli* (P22565). The plant and *Synechocystis* sequences are most similar, showing four conserved cysteine residues that might participate in the coordination of the iron–sulfur bridge proposed to be involved in catalysis (Seemann *et al.*, 2002; Wolff *et al.*, 2003). The position of one of these cysteine residues is not conserved in the bacterial protein (Figure 2), but it is possible that the cysteine at position 261 in the *E. coli* sequence might participate in the [4Fe-4S] coordination. The *E. coli* protein also lacks an N-terminal extension that is present in the rest of the HDR sequences (Figure 2). Part of this region is highly conserved in both plants and *Synechocystis*, but the most N-terminal section in the plant proteins is absent in *Synechocystis* and hardly conserved in plants (Figure 2). This plant-specific N-terminal extension is predicted by the ChloroP algorithm (Emanuelsson *et al.*, 1999) to comprise a signal sequence for plastid import, consistent with the subcellular localization of the MEP pathway in plants. The deduced cleavage site of the putative plastid targeting peptide is indicated in Figure 2. The mature plant proteins are predicted to contain the N-terminal stretch conserved in *Synechocystis* but absent from the *E. coli* protein, suggesting that its presence might be important for the activity or the stability of the HDR enzyme in photosynthetic organisms.

Accumulation of HDR *transcripts correlates with carotenoid biosynthesis during tomato fruit ripening and Arabidopsis seedling deetiolation*

As a first approach to investigate the involvement of HDR in the production of prenyl diphosphate precursors for the biosynthesis of carotenoids in plants, we analyzed the pattern of accumulation of *HDR* transcripts during tomato fruit ripening and *Arabidopsis* seedling deetiolation, two distinct developmental stages that involve high carotenoid production rates. RNA blot hybridization experiments using a probe specific for the tomato gene (*LeHDR*) showed that transcript levels increased during the transition from mature green to orange fruit and in further stages of ripening, reaching the highest levels in red ripe fruit (Figure 3a). Analysis of the pigment content of the same fruit samples used for RNA extraction (Figure 3b) confirmed that the activation pattern of *HDR* expression closely correlated with the accumulation of carotenoids which, together with chlorophyll degradation, characterizes the transition from chloroplasts to chromoplasts responsible for the change of fruit color from green (mature green) to red (ripe) in tomato (Bartley and Scolnik, 1995; Gillaspy *et al.*, 1993).

Carotenoid biosynthesis is also activated during seedling deetiolation (Giuliano *et al.*, 1993; von Lintig *et al.*, 1997; Park *et al.*, 2002). In this case, however, carotenoid accumulation involves a coordinated production of chlorophylls to

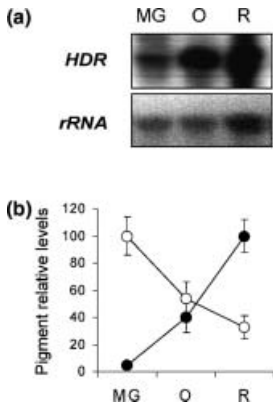

Figure 3. *HDR* expression and carotenoid accumulation during tomato fruit development. Tomato mature green (MG), orange (O), and ripe (R) fruit were ground in liquid nitrogen after removal of the seeds. The frozen powder was used for both RNA and pigment extraction.
(a) RNA blot analysis with the *LeHDR* probe. The same blot was also hybridized with a *25S rRNA* probe to monitor RNA loading.
(b) Total carotenoid (black circles) and chlorophyll (white circles) levels in the fruit samples expressed relative to their highest level. Mean and standard deviation of two independent extractions are indicated.

support photosynthetic metabolism. To confirm whether an induction of *HDR* expression was also required to support carotenoid biosynthesis during the light-induced transition from etioplasts to chloroplasts, RNA blot experiments were carried out with an *Arabidopsis HDR* gene-specific probe and RNA samples collected 0, 1 or 6 h after illumination of 3-day-old *Arabidopsis* dark-grown (etiolated) seedlings. RNA from light-grown seedlings of the same age was also analyzed. Figure 4(a) shows that *HDR* transcripts rapidly and strongly accumulated during deetiolation. Quantification of the radioactive signals from the blot (Figure 4b) showed that only 1 h after illumination the levels of HDR-encoding mRNAs had doubled compared with those measured right before the light signal. The amount of *HDR* transcripts after 6 h in the light was 10-fold higher than that in etiolated seedlings (Figure 4b), but it was still lower that the levels found in light-grown seedlings (Figure 4a). High-performance liquid chromatography (HPLC) analysis of the pigment content of the samples used for RNA extraction confirmed that the light signal also triggered a rapid activation of carotenoid biosynthesis (Figure 4c) and a concomitant increase in the production of chlorophylls (data not shown). The increase in total carotenoid levels was mostly due to the enhanced production of beta-carotene, a minor carotenoid in etioplasts but abundant in the thylakoid membranes of chloroplasts (Figure 4c). At 1 h after illumination the levels of beta-carotene were twofold higher than those measured in etiolated seedlings, and they almost doubled again at 6 h (Figure 4d). Together, a correlation between *HDR* expression and carotenoid biosynthesis was observed during both tomato fruit development and *Arabidopsis* seedling

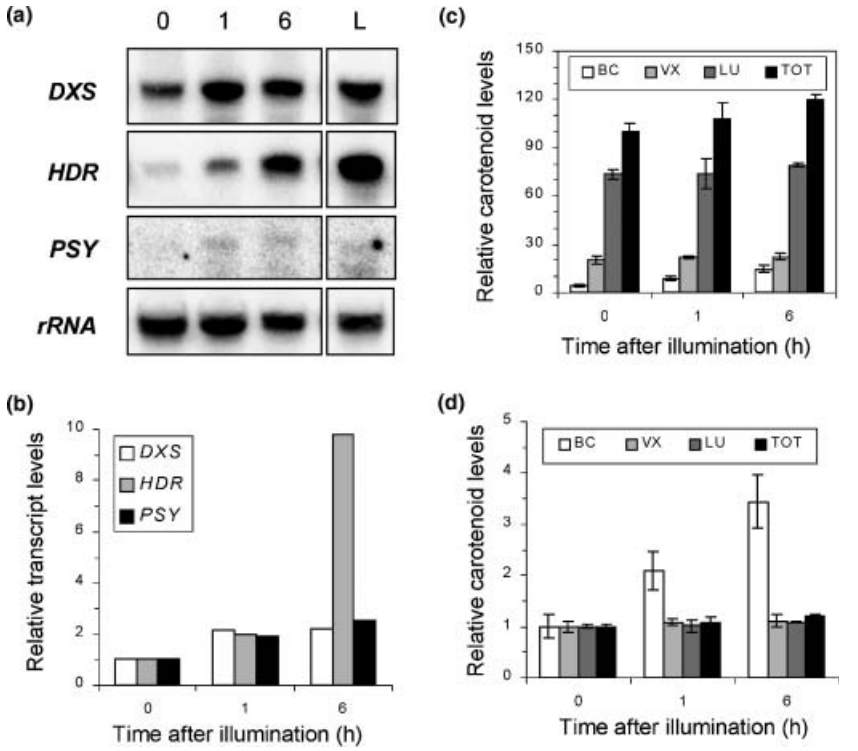

Figure 4. *HDR* expression and carotenoid accumulation during seedling deetiolation in *Arabidopsis*. Columbia-7 seeds were germinated on MS plates and incubated either under LD conditions or in the dark for 3 days. At time 0, samples of light-grown (L) and dark-grown seedlings were collected. The rest of etiolated seedlings were exposed to light and samples were taken after 1 and 6 h. Immediately after harvesting, samples were frozen in liquid nitrogen and ground to a fine powder that was later used for both RNA and carotenoid extraction.
(a) RNA blot analysis with *Arabidopsis* gene-specific probes to detect *DXS*, *HDR* and *PSY* transcripts. A *25S rRNA* probe was also used to monitor RNA loading. Blots were exposed for either 75 min (*HDR* and *rRNA*) or 2 days (*DXS* and *PSY*).
(b) Quantification of the radioactive signals from the blots shown in (a). Transcript levels are normalized with the *25S rRNA* data and represented relative to the amount measured at time 0.
(c and d) Carotenoid amounts in the samples used for RNA blot analysis relative to total carotenoid levels at time 0 (c) or to the levels of each carotenoid at time 0 (d). Results are shown for beta-carotene (BC), violaxanthin (VX), lutein (LU), and total carotenoids (TOT).

deetiolation, suggesting coordination between the regulation of *HDR* expression and the production of carotenoids in plant cells.

HDR *upregulation parallels that of DXS and PSY at early stages of carotenoid accumulation*

The upregulation of the *LeHDR* gene in the early stages of tomato fruit ripening (Figure 3a) was similar to that reported for genes encoding the enzymes DXS and phytoene synthase (PSY) (Lois *et al.*, 2000), which catalyze the first step of the MEP pathway and the first committed step for the biosynthesis of carotenoids respectively (Figure 1). Unlike *HDR*, however, the level of transcripts encoding DXS and PSY1 (the fruit isoform of PSY) was not increased (but even decreased) in ripe fruit, when carotenoid levels are highest (Lois *et al.*, 2000). To compare the pattern of expression of these three genes during the early stages of *Arabidopsis* seedling deetiolation, the same RNA samples used to analyze *HDR* transcript accumulation were used to prepare a replica blot which was then hybridized with specific probes to detect transcripts encoding DXS and PSY (Figure 4a). The exposure time required to detect *HDR* transcripts (about 1 h) was much shorter than that for *DXS* and *PSY* (2 days), suggesting that *HDR* gene expression level was dramatically higher at all time points tested. The rate of light-triggered induction of gene expression at short times was similar for all three genes. As shown in Figure 4(b), the amount of transcripts from both *DXS* and *PSY* genes virtually doubled in the first hour following illumination of dark-grown seedlings, similarly to that described for *HDR* expression. At 6 h, however, the strong upregulation of *HDR* gene expression was not paralleled by *DXS* and *PSY* transcript accumulation, which hardly increased (Figure 4b). The results suggest that the upregulation of *HDR* expression might be coordinated with that of other genes controlling carotenoid biosynthesis such as *DXS* and *PSY* at the onset of carotenoid production. At later stages, however, *DXS* and *PSY* upregulation appears to be attenuated whereas *HDR*-specific regulatory mechanisms are likely involved in further increasing transcript accumulation.

Arabidopsis seedlings overexpressing HDR show increased carotenoid levels in chloroplasts but not in etioplasts

To confirm whether enhanced HDR activity resulted in increased carotenoid production, we engineered *Arabidopsis* plants to overproduce a functional HDR protein under the control of the constitutive CaMV *35S* promoter and studied the derived effects on the biosynthesis of carotenoids. An *LeHDR* cDNA encoding the tomato full-length enzyme was used instead of the *Arabidopsis* sequence to prevent co-suppression. T_2 seeds from lines showing only one T-DNA insertion were used to analyze the expression level of the transgene. RNA blot analysis with an *LeHDR*-specific probe (encompassing the 5′-UTR region of the tomato gene, which is not conserved in *Arabidopsis*) showed that lines LH5, LH12, and LH13 expressed the transgene (Figure 5a).

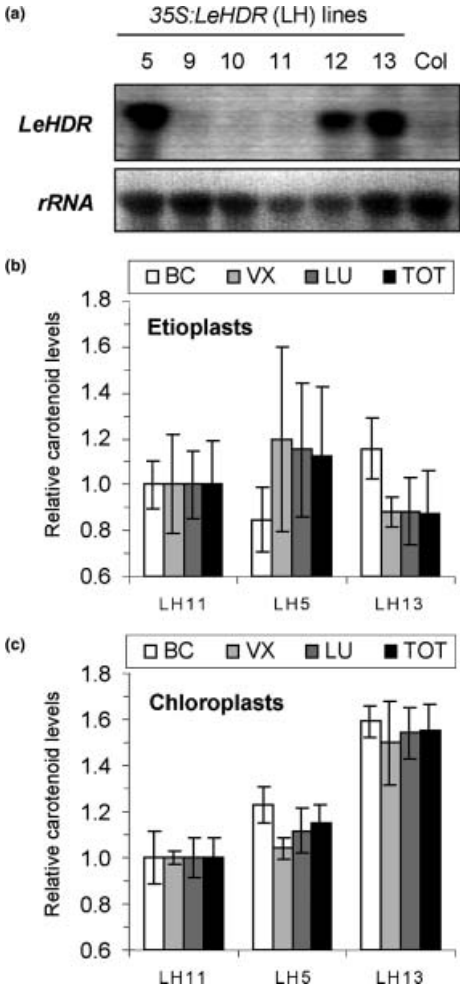

Figure 5. Analysis of *Arabidopsis 35S:LeHDR* transgenic lines.
(a) RNA blot analysis of *35S:LeHDR* T_2 seedlings with the tomato gene-specific *LeHDR* probe. RNA from untransformed Columbia-7 (Col) seedlings was included to test the level of cross-hybridization with the endogenous *Arabidopsis HDR* gene. A *25S rRNA* probe was also used to monitor loading.
(b) Quantification of carotenoid levels in 3-day-old etiolated seedlings of the indicated *35S:LeHDR* lines.
(c) Quantification of carotenoid levels in transgenic 3-day-old seedlings grown under LD conditions. Levels of beta-carotene (BC), violaxanthin (VX), lutein (LU) and total carotenoids (TOT) in (b) and (c) are represented relative to the amounts in LH11 seedlings. The mean and standard deviation shown correspond to two independent experiments with two replicas each.

Polymerase chain reaction (PCR) analysis of the lines with no detectable levels of tomato *HDR* transcripts showed that the transgene was disrupted in line LH11 (data not shown). For further experiments, we selected the overexpressing lines LH5 and LH13 (which showed the highest levels of transgene expression), and line LH11 as a control. The levels of carotenoids in T_2 populations of etiolated and light-grown seedlings were measured by HPLC. No significant differences in the accumulation of carotenoids were found in dark-grown LH5 and LH13 seedlings compared with the control line LH11 (Figure 5b) or wild-type etioplasts (data not shown). By

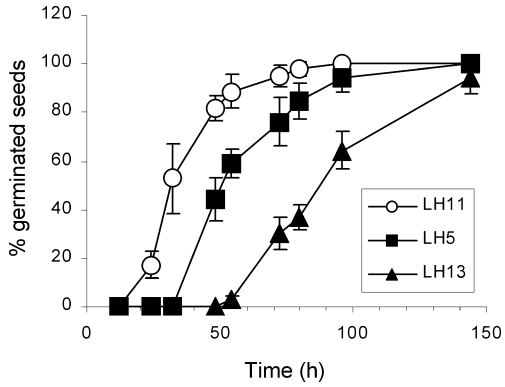

Figure 6. Seed dormancy phenotype of *35S:LeHDR* plants. Seeds from the transgenic lines LH11, LH5, and LH13 were germinated on MS plates after cold treatment. Germination was scored when at least one cotyledon was visible. Mean and standard deviation of three separate experiments are shown.

contrast, when LH13 seedlings were grown under a long-day (LD) light regime (16 h light and 8 h dark) they produced ca. 50% higher levels of the major chloroplast carotenoids beta-carotene, lutein, and violaxanthin compared with those in plants that did not express the *LeHDR* transgene. (Figure 5c). Although the mean amount of carotenoids measured in LH5 seedlings tended to be higher than in control plants, the differences were lower than those observed in LH13 plants. The LH5 line, however, did show a significantly increased production of beta-carotene compared with control seedlings (Figure 5c).

Seed germination is delayed in Arabidopsis lines overexpressing HDR

Besides the higher levels of carotenoids detected in the *LeHDR*-overexpressing plants, we observed that seeds from both LH5 and LH13 lines germinated more slowly than control seeds that had been similarly collected and stored. Figure 6 shows the quantification of this phenotype. Following a pattern that paralleled carotenoid accumulation, line LH13 showed the strongest dormancy phenotype. Germination of LH5 seeds was also significantly delayed compared with LH11 (Figure 6). As carotenoids are the precursors for the synthesis of ABA, a hormone with a key role in promoting seed dormancy (Schwartz *et al.*, 2003; Zeevaart and Creelman, 1988), it is possible that the enhanced production of carotenoids by LH13 and LH5 plants is afterwards channeled to the biosynthesis of ABA in transgenic seeds.

Synthesis of other GGPP-derived isoprenoid products is boosted after HDR overexpression

To confirm that the observed increase in carotenoid levels of *35S:LeHDR* plants was due to the enhanced production of

their metabolic precursors, we studied the effects of an activated *HDR* expression in the biosynthesis of other GGPP-derived isoprenoids. As the accumulation of at least some isoprenoid end products might be prevented by regulatory mechanisms specific of their biosynthetic pathways (Estévez *et al.*, 2001), we monitored the production of taxadiene, a non-native plastidial isoprenoid in *Arabidopsis* that is directly synthesized from GGPP by the activity of taxadiene synthase (TXS). This plastidial enzyme catalyzes the first committed step in the biosynthesis of paclitaxel (also known as Taxol), a powerful anti-cancer drug produced by the yew tree (Koepp *et al.*, 1995). Transgenic *Arabidopsis 35S:TXS* plants constitutively overexpressing a TXS-encoding cDNA from *Taxus baccata* are able to synthesize taxadiene, although at relatively low levels (Besumbes *et al.*, 2004). To estimate whether an enhanced production of isoprenoid precursors through the MEP pathway resulted in higher rates of taxadiene biosynthesis, *35S:TXS* plants were crossed with plants overexpressing either DXS (Carretero-Paulet, 2003) or HDR (line LH13). As a control, *35S:TXS* plants (in the Columbia-3 background) were crossed with wild-type plants of the Columbia-7 background (the one used for the generation of *35S:DXS* and *35S:LeHDR* plants). At least three F_1 plants from each of the three crosses were allowed to self-cross. Seedlings carrying at least one copy of each parental transgene in their genome were selected from the resulting F_2 segregating populations and used for experiments. To test the activity of the constructs, we compared the levels of carotenoids in the selected seedlings. As shown in Figure 7(a), seedlings from the TXS + DXS and TXS + HDR crosses contained ca. 50% higher carotenoid levels than those derived from the cross with the wild-type, therefore confirming that both *35S:DXS* and *35S:LeHDR* constructs remained active and that an increased activity of the corresponding proteins results in enhanced production of carotenoids. When samples were used to measure taxadiene content by GC-MS, a 6.5-fold and 13-fold higher increase in the accumulation of taxadiene was detected in TXS + DXS and TXS + HDR seedlings, respectively, compared with control seedlings carrying only the *35S:TXS* transgene (Figure 7b). These results highlight the key role of both DXS and HDR enzymes in controlling the production of GGPP and suggest a major contribution of HDR to the metabolic regulation of plastidial isoprenoid biosynthesis.

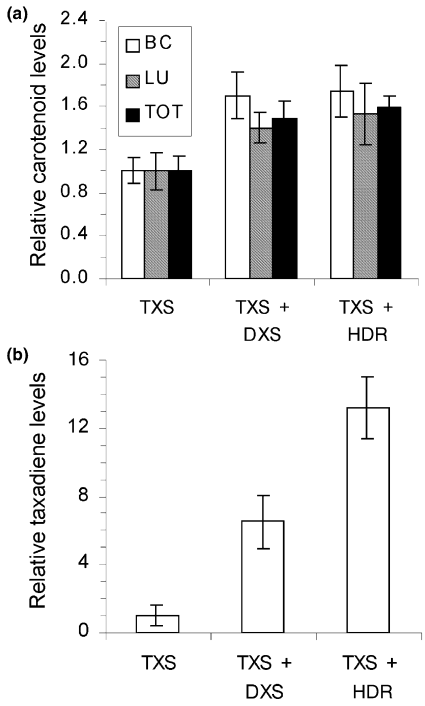

Figure 7. Carotenoid and taxadiene production in double transgenic plants. F_2 seeds obtained after crossing Columbia-3 *35S:TXS* plants with Columbia-7 wild-type (TXS), *35S:DXS* (TXS + DXS), or *35S:LeHDR* (TXS + HDR) plants were germinated on plates to select the parental markers and grown under LD conditions. At day 10, two pools of seedlings from each population were collected, frozen in liquid nitrogen, and ground to a fine powder for extraction of carotenoids and taxadiene.
(a) Levels of beta-carotene (BC), lutein (LU), and total carotenoids (TOT) relative to those in the TXS sample.
(b) Levels of taxadiene relative to those in the TXS sample. Mean and standard deviation of two separate extractions from each pool are shown.

Discussion

Carotenoids are the main group of plastidial isoprenoids synthesized in plants and enhancing their production is an important biotechnological goal due to their industrial and nutritional value (Bartley and Scolnik, 1995; Fraser and Bramley, 2004; Hirschberg, 2001; Sandmann, 2001). Until recently, the PSY-catalyzed production of phytoene from GGPP (Figure 1) was considered to be the first limiting step in the production of carotenoids. Studies carried out after the discovery of the MEP pathway, however, suggested that the rate-limiting nature of PSY activity was dependent on the availability of non-limiting levels of MEP-derived precursors (Lois *et al.*, 2000). The supply of plastidial precursors regulates the production of other isoprenoids such as chlorophylls, tocopherol, and gibberellins (Estévez *et al.*, 2001; Fray *et al.*, 1995). In fact, a major unsolved problem for the biotechnological production of high levels of carotenoids in plants is how to increase the flow of metabolic precursors to carotenoid synthesis without affecting other interacting metabolic pathways (Sandmann, 2001). To address this challenge, our research efforts focus on the identification of the control points in the MEP pathway as targets to manipulate and increase the production of IPP and DMAPP, the universal precursors of isoprenoids, in plastids. In this work we present evidence that the production of IPP and DMAPP catalyzed by the HDR enzyme represents a key step in controlling the production of plastidial isoprenoid precursors and carotenoid biosynthesis in plant cells.

A correlation between *HDR* transcript levels and carotenoid accumulation was observed in tomato fruit during ripening (Figure 3) and *Arabidopsis* seedlings during deetiolation (Figure 4). In the latter case, however, the pattern of *HDR* expression correlated with the production of beta-carotene rather than total carotenoids. Beta-carotene, together with lutein, is the major carotenoid species in the chloroplasts of *Arabidopsis* (Park *et al.*, 2002; Pogson *et al.*, 1996) and most other plants (Demmig-Adams *et al.*, 1996). By contrast, dark-grown (etiolated) *Arabidopsis* seedlings mostly synthesize the xanthophylls lutein and violaxanthin (Park *et al.*, 2002) (Figure 4c). Our data show that beta-carotene is the main carotenoid synthesized during *Arabidopsis* deetiolation, doubling its amount in only 1 h after illumination and further increasing afterwards (Figure 4d). Interestingly, beta-carotene also showed the highest increase in the *LeHDR*-overexpressing lines LH5 and LH13 (Figure 5b) and in both TXS + DXS and TXS + HDR double transgenic plants (Figure 7). These results suggest that the carotenoid pathway enzymes leading from IPP and DMAPP to beta-carotene are capable of readily converting an enhanced supply of substrate into product, at least in chloroplasts. We also observed that the high carotenoid levels of *35S:LeHDR* transgenic lines correlated with a delayed germination phenotype (Figure 6), strongly suggesting that part of the beta-carotene synthesized in these plants is channeled to the production of ABA (Figure 1), a carotenoid-derived hormone which plays a major role in maintaining seed dormancy (Schwartz *et al.*, 2003; Zeevaart and Creelman, 1988). Increased ABA levels and delayed germination of seeds have also been observed when carotenoid production was increased in *Arabidopsis* plants by overexpression of *DXS* (Estévez *et al.*, 2001) or *PSY* (Lindgren *et al.*, 2003). The results described here, in addition to previous reports showing that the inhibition of carotenoid biosynthesis in tomato fruit with the MEP pathway inhibitor fosmidomycin resulted in reduced seed dormancy (Rodríguez-Concepción *et al.*, 2001), further support that ABA biosynthesis depends on the activity of the MEP pathway and the production of carotenoids (at least in seeds).

These observations highlight the importance of understanding how the MEP pathway is coordinated with downstream pathways (and how these are coordinated among themselves) to make sure that the required precursors will be supplied when needed. Expression of MEP pathway genes either precedes or parallels the activation of specific pathways for the production of different isoprenoid end products, including carotenoids (Bouvier *et al.*, 1998; Hans *et al.*, 2004; Lange *et al.*, 1998; Lois *et al.*, 2000; Veau *et al.*, 2000; Walter *et al.*, 2000). Furthermore, changes in the levels of MEP pathway intermediates and carotenoid end products control the expression of *DXS* and *PSY1* in tomato fruit (Lois *et al.*, 2000; Rodríguez-Concepción *et al.*, 2001). These results support a cross-talk between the MEP pathway and the carotenoid pathway through the control of the expression of key genes, which may contribute to a fine regulation of carotenoid accumulation. The data reported here support this hypothesis, suggesting that *HDR* might be coordinately upregulated with *DXS* and *PSY* when an enhanced synthesis of carotenoids is required during the transition from mature green to orange fruit in tomato (Figure 3). A coordinated upregulation of *DXS*, *HDR*, and *PSY* gene expression also appears to take place during the very first stages of light-induced carotenoid accumulation in *Arabidopsis* seedlings (Figure 4). Interestingly, the *Arabidopsis HDR* gene (At4g34350) harbors an ATCTA motif at position -754 relative to translational start which is also present in the promoters of genes upregulated during deetiolation, including *DXS* and *PSY* (Welsch *et al.*, 2003). It is therefore possible that this motif participates in the rapid and coordinated induction of the three genes during dark to light transition in *Arabidopsis* seedlings. At later stages, *HDR* transcript levels significantly increase unlike those of *DXS* and *PSY* (Figure 4). Similarly, the highest levels of *HDR* transcripts in tomato fruit were found at the end of ripening (Figure 3), when the accumulation of mRNAs encoding DXS and PSY decreases compared with orange fruit (Lois *et al.*, 2000). While this may simply reflect greater stability of the *HDR* transcript, it may also indicate that distinct regulatory mechanisms modulate the expression of these genes once the biosynthesis of carotenoids has been activated.

Consistent with the role of carotenoids in light harvesting and photoprotection, light regulates carotenoid biosynthesis in plants at both transcriptional and post-transcriptional levels (Giuliano *et al.*, 1993; von Lintig *et al.*, 1997; Welsch *et al.*, 2000, 2003; Woitsch and Römer, 2003). The expression of the MEP pathway genes is also regulated by light (Carretero-Paulet *et al.*, 2002; Estévez *et al.*, 2000; Mandel *et al.*, 1996; Rodríguez-Concepción *et al.*, 2004). Recent results showing that DXR, HDS, and HDR (Figure 1) are targets of thioredoxin, a member of the ferredoxin/thioredoxin system (Balmer *et al.*, 2003; Lemaire *et al.*, 2004), suggest that besides upregulating gene expression light might also activate the metabolic flux through the MEP pathway by increasing the activity of at least some enzymes through thioredoxin-mediated reduction. A light requirement for HDR activity would explain why dark-grown transgenic seedlings overexpressing HDR do not accumulate more carotenoids than control seedlings. Alternatively, the low activity of PSY in etioplasts (Welsch *et al.*, 2000) might prevent the channeling of increased levels of prenyl diphosphates to carotenoid production in transgenic etiolated seedlings. Another possibility to explain why HDR overexpression only led to enhanced carotenoid biosynthesis in chloroplasts is that etioplast carotenoids might be synthesized from MVA-derived GGPP (or another cytosolic prenyl diphosphate downstream of IPP and DMAPP). This is

consistent with our recent results suggesting that MVA-derived prenyl diphosphates might be imported into the etioplast for the biosynthesis of plastidic isoprenoids (including carotenoids) in dark-grown *Arabidopsis* seedlings, whereas a phytochrome-dependent signaling pathway would block such exchange in light (Rodríguez-Concepción *et al.*, 2004).

Overexpression studies in *Arabidopsis* led to the conclusion that DXS is a limiting enzyme for the biosynthesis of plastidial isoprenoids in plant cells (Estévez *et al.*, 2001), but the relative contribution of other MEP pathway enzymes to the supply of precursors remain to be investigated. Here we show that increased levels not only of DXS but also of HDR lead to higher isoprenoid (carotenoid and taxadiene) levels in transgenic plants, suggesting that the assignment of a single rate-limiting step in the MEP pathway might not be appropriate in a metabolic context. Indeed, it is commonly understood from metabolic control analysis (MCA) that several enzymes may share control over the flux of a pathway, with different enzymes exhibiting different degrees of control (Fell, 1992; Thomas and Fell, 1998). In the case of carotenoids, PSY exhibits the highest flux control coefficient among the enzymes of the biosynthesis pathway downstream of GGPP (Fraser *et al.*, 2002), but the individual overexpression of other enzymes such as lycopene beta-cyclase also results in increased levels of total carotenoids (Ronen *et al.*, 2000; Rosati *et al.*, 2000). In addition, increased PSY activity levels in transgenic plants overexpressing the bacterial enzyme significantly reduced the influence of this enzyme over flux through the pathway, shifting the metabolic flux control to another step (Fraser *et al.*, 2002). These results suggest that the carotenoid pathway can compensate for fluctuations in precursor/product equilibrium and redistribute the balance of control within the pathway. A similar scenario might be true for the MEP pathway. As described above, our earlier results suggest that *DXS* expression might be feedback-regulated by the accumulation of carotenoids and MEP pathway intermediates such as deoxyxylulose in tomato fruit (Lois *et al.*, 2000; Rodríguez-Concepción *et al.*, 2001), and it is possible that a differential feedback regulation might cause the distinct pattern of expression of *HDR* compared with *DXS* and *PSY* at later stages of carotenoid accumulation during tomato ripening and *Arabidopsis* deetiolation (Figures 3 and 4). Altered levels of MEP pathway intermediates after overexpression of DXS or HDR might therefore influence gene expression or enzyme activity at these and other steps of the pathway. A thorough MCA and a detailed study of the effect of the overexpression of each enzyme on the activity of the rest will be required to assign which step has a greater influence in regulating flux to IPP and DMAPP. Nonetheless, our results clearly show that HDR exerts significant control over the flux of the MEP pathway to plastidial isoprenoids and can be considered a 'rate-determining' enzyme (an MCA term used instead of 'rate-limiting' when changes in the concentration of an enzyme cause a change in the pathway flux). In conclusion, future attempts to efficiently increase metabolic flux through the MEP pathway to carotenoids and other plastidial isoprenoids should take into account the requirement to target multiple steps simultaneously, including HDR.

Experimental procedures

Plant growth conditions

Growth conditions of tomato (*Lycopersicon esculentum* Mill. cv. Ailsa Craig) plants and fruit development stages were as previously described (Lois *et al.*, 2000). *Arabidopsis thaliana* seeds were surface-sterilized and germinated on Petri dishes with solid Murashige and Skoog (MS) medium as described (Carretero-Paulet *et al.*, 2002). After stratification for at least 2 days at 4°C plates were incubated at 22°C in a growth chamber under long day (LD) conditions (16 h under fluorescent white light and 8 h in the dark). Etiolated seedlings were produced from Columbia-7 seeds on MS plates. Following stratification, a 1-h pulse of white light was given before incubation in the dark for 3 days. Seed dormancy was monitored with populations collected from different transgenic lines at the same time and stored dry at 4°C. Seeds were plated on MS and kept at 4°C in the dark for 3 days. After stratification, plates were transferred to a growth chamber at 22°C under LD conditions. Germination was considered complete when at least one cotyledon was visible.

Sequence analysis

Plant HDR homologues were searched using the *A. aestivalis* protein sequence (accession number AAG21984) as a query and the TBLASTN algorithm on the web page of TIGR (http://www.tigr.org). Plastid-targeting peptides were predicted with the ChloroP program online (http://www.cbs.dtu.dk/services/ChloroP). Sequence analyses were performed using the GCG 9.0 software package (Genetics Computer Group Inc.) and the available web resources (http://www.expasy.org).

RNA blot analysis

Tissue from tomato fruit pericarp or *Arabidopsis* whole seedlings was grounded in liquid nitrogen to a fine powder, part of which was used for total RNA extraction as described (Lois *et al.*, 2000). Electrophoresis, blotting, hybridization, and washes were carried out as described (Lois *et al.*, 2000). A Molecular Imager FX (Bio-Rad, Hercules, CA, USA) was used for exposure and quantification of the radioactive signals with the supplied Quantity One software. When indicated, the same filter was hybridized with several probes after stripping in 0.1% SDS at 100°C for 10 min. Probes were made by RT-PCR from total RNA as described (Rodríguez-Concepción *et al.*, 2004). Gene-specific primers were used to amplify cDNA sequences from tomato *HDR* (LeF1 5′-C G G G A T G C C T A A G G C T A A A C T C C-3′ and LeR2 5′-C T G G T C A G A T T C T T C C G-3′, which amplify 389 bp from the 5′-end of the cDNA) and *Arabidopsis HDR* (AtHPF 5′-C A T T G A A G T G A A C A G T C A G C-3′ and AtHPR 5′-T G T A C T C T C G A T T C A T G A G C-3′, which amplify 334 bp from the 5′-end of the cDNA) and *PSY* (AtYPF 5′-A G T A T G T C T T C T T C T G T A G C-3′ and AtYPR 5′-T A T T C A G C G C A A A C T T C A C C-3′, which

amplify 725 bp encoding the N-terminal region). The *DXS* and *25S rRNA* probes were made as described (Rodríguez-Concepción *et al.*, 2004). To confirm that the amplified PCR products corresponded to the target genes, direct sequencing of the PCR products with internal primers was carried out using the Big Dye Terminator Cycle Sequencing v2.0 kit (ABI-PRISM; PE Biosystems, Foster City, CA, USA).

Generation and analysis of single and double transgenic plants

A cDNA encoding the full-length tomato HDR protein was amplified from orange fruit cDNA by PCR with *Taq* DNA polymerase (Promega, Madison, WI, USA) and primers LeF1 (see above) and LeR1 (5′-G G T G G A A C C A A A T T G C A G C A C T C C-3′) designed from the sequence available on TIGR (TC124188). The 1.6 kb PCR product was cloned into the pGEM-T vector (Promega) and sequenced to confirm its identity. The entire sequence was amplified by PCR with *Pfu* DNA polymerase (Promega) and vector primers SP6 and T7, gel-purified, and cloned into the pCAMBIA 1303 vector previously digested with *Pml*I and *Bgl*II and blunt-ended with Klenow. In the resulting construct, the *LeHDR* cDNA was under the transcriptional control of the *CaMV 35S* promoter. Integrity of the constructs was checked by PCR with specific primers. Transgenic *Arabidopsis* plants harboring this *35S:LeHDR* construct were generated in the Columbia-7 background via *Agrobacterium*-mediated infiltration as described (Carretero-Paulet *et al.*, 2002). Plants expected to harbor the transgene were selected based on their ability to survive when grown on MS plates supplemented with 50 μg ml^{-1} hygromycin. The resulting positives (T_1 generation) were transferred to soil and allowed to set seed. T_2 seeds from lines showing only one T-DNA insertion (as determined by the Mendelian segregation of the hygromycin selection marker) were then selected for further experiments. A homozygous (T_3) plant (line LH13) was crossed with a homozygous *35S:TXS* plant (Besumbes *et al.*, 2004) to generate double transgenic plants. Similarly, *35S:TXS* plants were crossed with a homozygous *35S:DXS* line (Carretero-Paulet, 2003) and Columbia-3 wild-type plants. The F_1 generation was selected on MS plates based on the male parental marker and allowed to set seed on soil. F_2 populations of seedlings carrying at least one copy of each transgene were identified on plates supplemented with the appropriate antibiotics to select the parental markers and used for further experiments.

Isoprenoid measurement

Extraction, separation by HPLC, and quantification of chlorophylls (*a* and *b*) and carotenoids (violaxanthin, lutein, and beta-carotene) were carried out as described (Rodríguez-Concepción *et al.*, 2004). Taxadiene levels were determined by GC-MS as described (Besumbes *et al.*, 2004).

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