Enhanced flux through the methylerythritol 4-phosphate pathway in *Arabidopsis* plants overexpressing deoxyxylulose 5-phosphate reductoisomerase

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Abstract: The methylerythritol 4-phosphate (MEP) pathway synthesizes the precursors for an astonishing diversity of plastid isoprenoids, including the major photosynthetic pigments chlorophylls and carotenoids. Since the identification of the first two enzymes of the pathway, deoxyxylulose 5-phoshate (DXP) synthase (DXS) and DXP reductoisomerase (DXR), they both were proposed as potential control points. Increased DXS activity has been shown to up-regulate the production of plastid isoprenoids in all systems tested, but the relative contribution of DXR to the supply of isoprenoid precursors is less clear. In this work, we have generated transgenic *Arabidopsis thaliana* plants with altered DXS and DXR enzyme levels, as estimated from their resistance to clomazone and fosmidomycin, respectively. The down-regulation of DXR resulted in variegation, reduced pigmentation and defects in chloroplast development, whereas DXR-overexpressing lines showed an increased accumulation of MEP-derived plastid isoprenoids such as chlorophylls, carotenoids, and taxadiene in transgenic plants engineered to produce this non-native isoprenoid. Changes in DXR levels in transgenic plants did not result in changes in DXS gene expression or enzyme accumulation, confirming that the observed effects on plastid isoprenoid levels in DXR-overexpressing lines were not an indirect consequence of altering DXS levels. The results indicate that the biosynthesis of MEP (the first committed intermediate of the pathway) limits the production of downstream isoprenoids in *Arabidopsis* chloroplasts, supporting a role for DXR in the control of the metabolic flux through the MEP pathway.

Keywords: Arabidopsis; Carotenoids; Deoxyxylulose 5-phosphate reductoisomerase (DXR); Isoprenoid biosynthesis; Methylerythritol 4-phosphate (MEP) pathway; Taxadiene.

Introduction

Plants produce an astonishing diversity of isoprenoids, a functionally and structurally diverse group of com-pounds synthesized from the C5 precursors isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) (Chappell 1995; Croteau et al. 2000). Addition of IPP units to DMAPP leads to the synthesis of prenyl diphosphates of increasing size such as geranyl diphosphate (GPP, C10), farnesyl diphos-phate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20), which are the starting points for multi-ple branches leading to the final isoprenoid products (Fig. 1). Unlike most organisms, plants have two sep-arated pathways for IPP and DMAPP biosynthesis: the mevalonic acid (MVA) pathway, which produces cytosolic IPP, and the plastid-localized methylerythri-tol 4-phosphate (MEP) pathway (Lichtenthaler 1999; Eisenreich et al. 2001; Rodríguez-Concepció n and Boronat 2002). Despite plastidial isoprenoids including

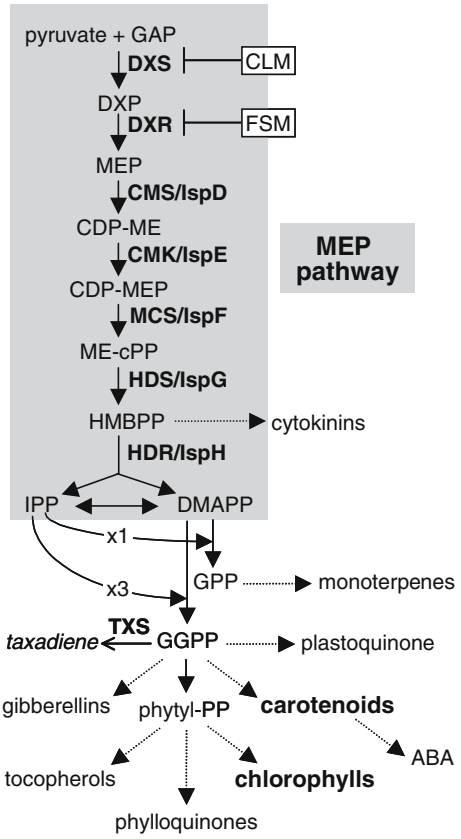

Fig. 1 The pathway for isoprenoid biosynthesis in plastids. GAP, glyceraldehyde 3-phosphate; DXP, deoxyxylulose 5-phosphate; MEP, methylerythritol 4-phosphate; CDP-ME, 4-diphosphocytidyl-methylerythritol; CDP-MEP, CDP-ME 2-phosphate; ME-cPP, methylerythritol 2,4-cyclodiphosphate; HBMPP, hydroxymethylbutenyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid. The MEP pathway enzymes are indicated in bold (the names of the bacterial homologues are also indicated): DXS, DXP synthase; DXR, DXP reductoisomerase; CMS, CDP-ME synthase; CMK, CDP-ME kinase; MCS, ME-cPP synthase; HDS, HMBPP synthase; HDR, HMBPP reductase. The step catalyzed by taxadiene synthase (TXS) is shown, as well as the steps inhibited by clomazone (CLM) and fosmidomycin (FSM)

the major photosynthetic pigments (chlorophylls and carotenoids) as well as other key photosynthesis-related compounds (plastoquinones, phylloquinones, and tocopherols), hormones (gibberellins and abscisic acid), and monoterpenes are synthesized from MEP-derived precursors (Fig. 1), the MEP pathway was only recently discovered and fully elucidated (Rodríguez-Concepción and Boronat 2002; Rohdich et al. 2003).

The initial reaction of the MEP pathway, catalyzed by deoxyxylulose 5-phosphate (DXP) synthase (DXS), involves the condensation of (hydroxyethyl)thiamin derived from pyruvate with the C1 aldehyde group of glyceraldehyde 3-phosphate (GAP) to produce DXP. In the second step, an intramolecular rearrangement and reduction of DXP by the enzyme DXP reductoisomerase (DXR) yields MEP, the first committed precursor of plastid isoprenoids. After conversion of MEP into hydroxymethylbutenyl diphosphate (HMBPP) in four enzymatic steps (Fig. 1), the enzyme HMBPP reductase (HDR) converts HMBPP into IPP and DMAPP in the last step of the MEP pathway (reviewed in Rodríguez-Concepción and Boronat 2002; Rohdich et al. 2003). Despite the impressive progress in the elucidation of the MEP pathway, relatively little is currently known on the contribution of the corresponding enzymes to control the flux of intermediates through the pathway and the supply of IPP and DMAPP for the synthesis of plastid isoprenoid end-products. Since the identification of the first two enzymes of the pathway (DXS and DXR), they both were tentatively proposed as potential control points. The role of these enzymes in plants has been often investigated using model systems in which high IPP and DMAPP levels are required to support an increased production of plastid isoprenoids in response to external stimuli or developmental cues. Thus, analysis of *DXS* expression patterns during accumulation of different plastidial isoprenoid end products suggested that increased DXS levels might be required to supply their precursors (Mandel et al. 1996; Bouvier et al. 1998; Lange et al. 1998; Chaded et al. 2000; Estévez et al. 2000; Lois et al. 2000; Veau et al. 2000; Walter et al. 2000; Walter et al. 2002; Burlat et al. 2004; Botella-Pavía et al. 2004). Transgenic *Arabidopsis* and tomato plants in which DXS activity had been altered confirmed the regulatory role of DXS in controlling flux through the MEP pathway (Estévez et al. 2001; Enfissi et al. 2005). In the case of DXR, a positive correlation between enhanced plastid isoprenoid biosynthesis and transcript accumulation has been observed in some plant systems (Walter et al. 2000; Veau et al. 2000; Carretero-Paulet et al. 2002; Hans et al. 2004; Hsieh and Goodman 2005a; Mayrhofer et al. 2005; Bede et al. 2006) but not in others (Rodríguez-Concepción et al. 2001; Dudareva et al. 2005). Overexpression of DXR in peppermint led to increased monoterpene levels in leukoplasts of the non-photosynthetic secretory cells of glandular trichomes (Mahmoud and Croteau 2001), but it is possible that the production of MEP from DXP might also limit the biosynthesis of other isoprenoids in other plastid types. In this work we have modified DXR activity levels in transgenic *Arabidopsis* plants to characterize its relative contribution to the production of primary (chlorophylls and carotenoids) and secondary (taxadiene) isoprenoid end-products in chloroplasts. The results demonstrate that DXR, together with other enzymes

of the MEP pathway, controls flux to IPP and DMAPP in photosynthetic tissues.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana seeds were surface-sterilized and germinated on Petri dishes with solid Murashige and Skoog (MS) medium as described (Rodríguez-Concepción et al. 2004). Unless otherwise stated, after stratification for at least 2 days at 4°C plates were incubated in a growth chamber at 22°C under long day (LD) conditions (8 h in the dark and 16 h under fluorescent white light at a photon fluence rate of 100 μmol m^{-2} s^{-1}). When indicated, the medium was supplemented with different concentrations of clomazone (CLM, a gift of Zeneca Agrochemicals) from a 0.1 M stock solution prepared in water, or fosmidomycin (FSM, Gateway Chemical Technology) prepared as described (Rodríguez-Concepción et al. 2001). Seedling establishment (SE, defined as the percentage of seedlings producing green true leaves that are photosynthetically active and therefore able to support full plant development) was monitored for each seed stock on MS plates supplemented or not with CLM or FSM. The SE rate in the presence of the inhibitor was calculated relative to the value observed on plates without it (which was considered as 100%). For seed production, plants were transferred from the plates to a 1:1:1 (v/v) perlite:vermiculite:sphagnum soil mixture irrigated with mineral nutrients (Carretero-Paulet et al. 2002) and grown in the LD chamber.

Overexpression constructs

Transgenic *Arabidopsis* lines overexpressing DXS were generated in the Columbia-3 background using a construct previously used for transformation of Columbia-7 plants (Botella-Pavía et al. 2004). Briefly, a cDNA encoding the full-length *Arabidopsis* DXS protein was amplified by PCR with *Pfu* DNA polymerase (Promega) and primers DXS.*BamHI* (5′-CAGGATCCCATTTGTTTTACTGTTTTTGATAACC-3′) and DXS.*SacI* (5′-CAGAGCTCCACTGCTCGTACCAAAATATGTC-3′) using as a template the EST clone H2A12T7 (provided by the Nottingham Arabidopsis Stock Centre). The PCR product was digested with *BamHI* and *Sac*I and cloned into the same sites of plasmid pBI121 (Clontech). In the resulting construct (designated p35S:DXS), the transcription of the cDNA encoding DXS was controlled by the cauliflower mosaic virus *35S* promoter. A similar strategy was followed to generate the p35S:DXR construct to constitutively overexpress the *Arabidopsis* cDNA encoding DXR. A cDNA amplified by PCR using the pDXR-At plasmid (Carretero-Paulet et al. 2002) as a template with *Pfu* DNA polymerase (Promega) and primers DXR.XbaI (5′-CTTCTAGACAAGAGTAGTAGTGCGGTTCTCTGG-3′) and DXR.SacI (5′-CAGAGCTCCAGTTTGGCTTGTTCGGATCACAG-3′) was digested with *Xba*I and *Sac*I and cloned into the same sites of pBI121 to create plasmid p35S:DXR. The integrity of the constructs was confirmed by DNA sequencing with the Big Dye Terminator Cycle Sequencing v1.1 kit of the ABI-PRISM system (PE Biosystems).

Generation of transgenic plants

Arabidopsis plants of the Columbia-3 background were transformed with the p35S:DXS and p35S:DXR constructs via *Agrobacterium*-mediated infiltration as described (Carretero-Paulet et al. 2002) and designated S (*35S:DXS*) and R (*35S:DXR*) lines. Plants expected to harbor the transgene were selected based on their ability to survive when grown on MS plates supplemented with 50 μg/ml kanamycin. The resulting positives (T_1 generation) were transferred to soil and allowed to set seed. Segregation of the kanamycin selection marker allowed to identify the lines harboring only one T-DNA insertion and to obtain homozygous T_3 plants. To generate double transgenic plants, ovaries of transgenic S-5 and R-2 plants overexpressing DXS and DXR, respectively, were fertilized with pollen from a homozygous *35S:TXS* plant of the C2 line (Besumbes et al. 2004). Wild-type ovaries were also pollinated with *35S:TXS* pollen as a control. The F_1 generation was selected on MS plates supplemented with 50 μg/ml hygromycin (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 hygromycin and kanamycin to select the parental markers and used for further experiments.

Analysis of transcript and protein levels

Total RNA was isolated using the RNAeasy plant mini kit (Qiagen). On-column DNaseI digestion was performed following the manufacturer's protocol to prevent contamination with genomic DNA. A Bioanalyzer 2100 (Agilent Technologies) was used to assess the amount and quality of the RNA. For RNA blot

analysis, 20 µg of RNA from each sample were fractionated by electrophoresis in a 1% (w/v) agarose gel containing 2.2 M formaldehyde and blotted onto a Hybond N^+ nylon membrane (Amersham). The blot was hybridized for 18 h at 68°C in ExpressHyb solution (Clontech) with a *DXR* probe obtained from a 1.3 kb cDNA fragment released from the pDXR-At vector (Carretero-Paulet et al. 2002) by *Eco*RV digestion and labeled with ^{32}P-dCTP using the Ready-to-Go kit (Pharmacia). Washes were performed at 50°C three times in 2× SSC, 0.05% SDS, and twice in 0.1× SSC, 0.1% SDS. Biomax (Kodak) film was used for exposure with intensifying screens for 3 days at –80°C.

For real-time quantitative reverse transcription PCR, equal amounts of total RNA from two independent samples were combined and the RNA mixture was retrotranscribed with TaqMan reverse transcription reagents (Applied Biosystems). Triplicate PCR reactions were performed in the iCycler thermocycler (Biorad) using TaqMan Universal PCR Master Mix, No AmpErase, UNG (Applied Biosystems), cDNA derived from 20 ng of input RNA, and predesigned FAM-labeled TaqMan MGB probes and unlabeled primers (Applied Biosystems) for DXS (At02221413_g1), DXR (At02281385_g1), and EF1α (elongation factor –1α, At02337969_g1) to normalize the threshold cycle (C_T) for each probe assay. Relative gene expression was estimated as $2^{-\Delta C}{}_T$ and fold change was estimated by the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

Protein extraction and immunoblot analysis were performed as described (Carretero-Paulet et al. 2002). Scanned immunoblot images were used for quantification of DXS and DXR protein levels with the Quantity One software (Biorad). The levels of two abundant proteins of the same extracts (detected by Coomassie staining) were also quantified for normalization.

Quantification of plastid isoprenoids

Chlorophyll and carotenoid pigments were extracted and separated by HPLC on a Waters Alli-ance 2690 system using a reverse-phase YMC 5 µm (250 × 4.6 mm) C_{30} column (Rodríguez-Concepció n et al. 2004). Eluting compounds were monitored using a Waters 2996 Photodiode Array detector. Peak areas of chlorophylls (chlorophyll *a* and chlorophyll *b*) and major carotenoids (lutein and *β*-carotene) at 450 nm were compared to that of a canthaxanthin internal standard for quantification using the Waters Millennium v.3.2 software supplied. Taxadiene levels were determined by GC-MS as described (Besumbes et al. 2004).

Microscopy

Chlorophyll autofluorescence in whole plants was observed by confocal laser scanning microscopy images with a Leica TCS 4D microscope using a LP590 filter after excitation with green light at 568 nm. For transmission electron microscopy, leaf samples from plants grown on MS plates for 12 days under LD conditions were collected. Cross-sections (1–2 mm wide) were fixed at reduced pressure in 2.5% glutaraldehyde and 2% paraformaldehyde in 100 mM sodium cacodylate buffer (pH 7.4) for 2 h at 4°C. Following four washes for 10 min at 4°C in cacodylate buffer, samples were post-fixated in 1% buffered OsO_4 for 1 h, rinsed in water, dehydrated in a gradient of acetone, and embedded in Spurr's resin at –60°C. After microtomy, ultrathin sections were stained with 2% uranyl acetate for 30 min followed by 2.6% lead citrate for 10 min. Specimens were observed with a Hitachi H800 MT transmission electron microscope and images were recorded using the Digital Micrograph 3.3.0 (Gatan Ltd.) software.

Results

Generation and analysis of 35S:DXR plants

In order to evaluate whether changes in DXR activity levels might influence the metabolic flux of the MEP pathway for the biosynthesis of isoprenoid precursors in chloroplasts, we engineered *Arabidopsis* plants to constitutively overproduce the enzyme. A construct containing the full-length cDNA encoding *Arabidopsis* DXR under the transcriptional control of the cauliflower mosaic virus *35S* promoter was transferred to *Arabidopsis* (Columbia-3) plants via *Agrobacterium*. Transgenic plants were selected based on kanamycin resistance and designated R (*35S:DXR*) lines. Fifteen independent R lines showing only one T-DNA insertion were selected and homozygous T_3 populations of these lines were used for experiments. Similarly, *Arabidopsis* plants constitutively overexpressing DXS were generated in the same background and designated S (*35S:DXS*) lines. Two S lines showing high levels of transgene expression (S-5 and S-6, data not shown) were selected as controls.

Most *35S:DXR* plants were virtually undistinguishable from the wild-type (Fig. 2A, B). In five lines, however, paler seedlings and variegated plants with green and albino sections and a developmental delay were observed (Fig. 2C, D). Variegation was observed in most individuals of line R-3 (Fig. 2D), whereas it

was only occasionally observed in other lines, including R-1 (Fig. 2C). In addition, not all the individuals from homozygous populations were affected to the same degree, consistent with variegation being caused by transgene-mediated gene silencing. The variegated phenotype was first obvious on the first emerging true leaves (Fig. 2C, D) but it could affect all tissues and organs, including mature leaves (Fig. F, G), inflorescence stems (Fig. 2H), flowers (Fig. 2I), and fruit (Fig. 2J).

Variegation correlated with the existence of different leaf sectors with a range of chloroplast abundance (Figs. 2K–N). To investigate whether the phenotype of variegated plants was also associated with defects in chloroplast development, different sections of variegated leaves from 12-day-old R-3 plants were observed using transmission electron microscopy (Fig. 3). Analysis of the ultrastructure of mesophyll plastids showed that green sectors contained lens-shaped chloroplasts similar to those observed in wild-type leaves (Fig. 3A, B). In the transition to white areas, more rounded chloroplasts with an underdeveloped thylakoid membrane system were located (Fig. 3C). Chloroplasts from pale sections showed a few loosening thylakoid membranes and lacked grana (Fig. 3D), whereas albino tissues contained plastids with only vesicle-like structures (Fig. 3E) and electrodense spherical bodies (Fig. 3F). This plastid phenotype is similar to that found in plants with silenced genes for MEP pathway enzymes acting upstream (DXS) or downstream (MCS, HDS, HDR) of DXR (Mandel et al. 1996; Estévez et al. 2000; Nagata et al. 2002; Gutierrez-Nava et al. 2004; Page et al. 2004; Hsieh and Goodman 2005a, b),

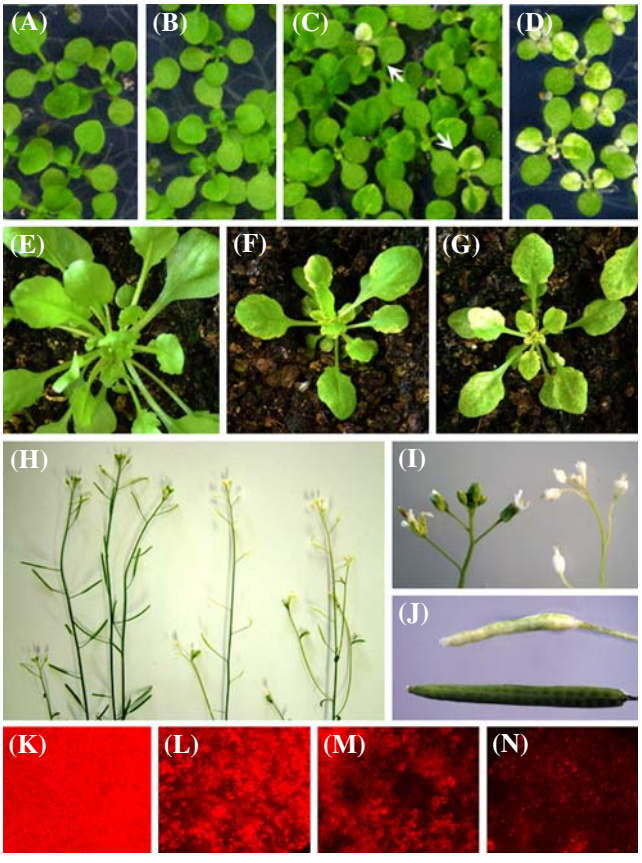

Fig. 2 Phenotype of 35S:DXR plants. (**A**) 15-day-old untransformed plants grown on MS. (**B**–**D**) 15-day-old R-2 (**B**), R-1 (**C**), and R-3 (**D**) plants grown on MS. Arrows mark variegated individuals from homozygous R-1 populations. (**E**) One-month-old untransformed plant on soil. (**F**–**G**) Variegated R-1 (**F**) and R-3 (**G**) plants grown as the one in (**E**). (**H**–**J**) Inflorescences (**H**), flowers (**I**) and fruit (**J**) from untransformed (green) and R-3 plants. (**K**–**N**) Chlorophyll autofluorescence of R-3 leaf areas with a green (**K**), slightly variegated (**L**), strongly variegated (**M**) and virtually albino (**N**) phenotype as observed with confocal laser-scanning microscopy

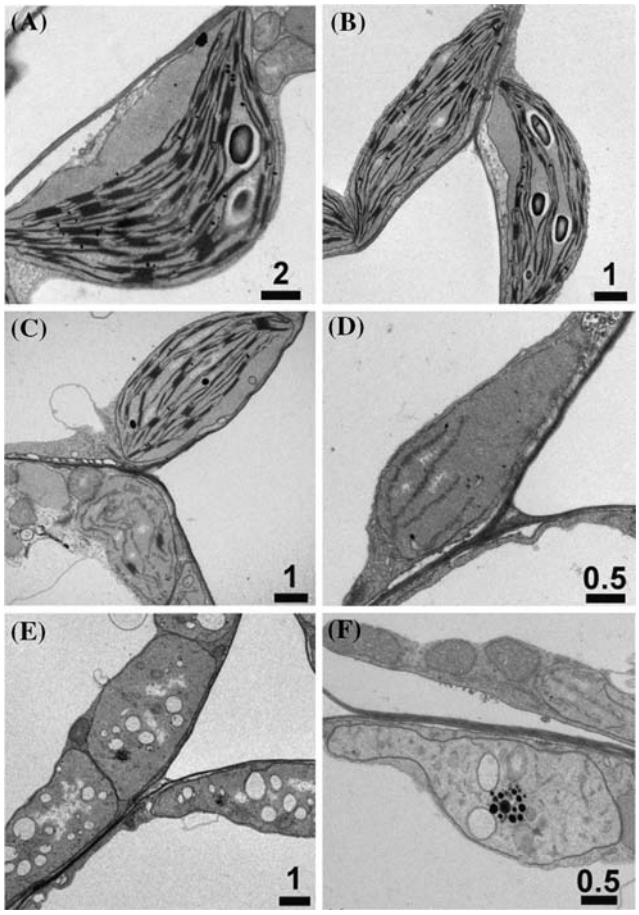

Fig. 3 Ultraestructural analysis of R-3 mesophyll plastids. The first true leaves of 12-day-old plants were collected and used for electron microscopy. (**A**) Chloroplasts from untransformed plants. (**B**–**F**) Plastids from R-3 leaf sections with increasing pigmentation defects, from green (**B**) to albino (**F**). Numbers on the bars indicate length (in μm)

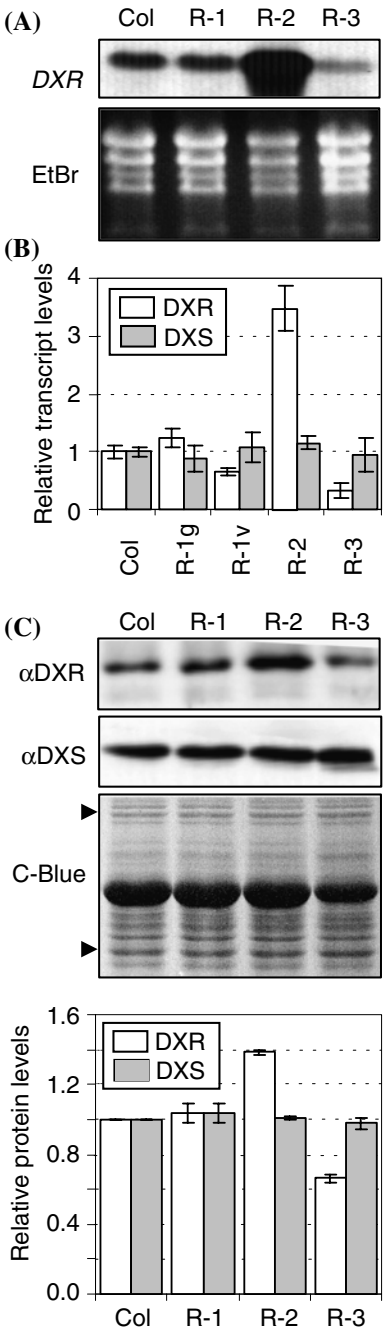

Fig. 4 Analysis of DXR and DXS levels in 35S:DXR plants. (**A**) RNA blot analysis. RNA was extracted from homozygous populations of 35S:DXR lines (R-1, R-2 and R-3) and untransformed (Col) plants grown on MS plates under LD conditions for 12 days and used for RNA blot analysis with a DXR gene-specific probe. Ethidium bromide (EtBr) staining is shown to compare loading. (**B**) Real time quantitative RT-PCR analysis of transcript levels in plants of the indicated lines grown as described in (**A**). In the case of the R-1 line, individuals with variegated leaves (R-1v) were collected separately from those that were fully green (R-1g). RNA from two independent experiments was combined, reversed transcribed, and used for triplicate PCR analysis. Mean and standard deviation of estimated fold change is shown. (**C**) Immunoblot analysis. Total protein samples isolated from similar samples as those described in (**A**) were separated by SDS-PAGE and analyzed with an anti-DXR serum (αDXR). A replica blot was incubated with an antibody against DXS (αDXS). Coomassie-blue (C-Blue) staining is shown to compare loading. Because R-3 plants showed a significantly lower accumulation of the RuBisCO large subunit (the major protein in these extracts), protein levels were normalized after those of two other abundant proteins detected by C-Blue staining (bands indicated with arrowheads). Mean and standard deviation of the two normalizations relative to Col levels are shown

suggesting that it is likely the result of an impaired production of plastidial IPP and DMAPP precursors.

To confirm whether a transgene-induced down-regulation of DXR levels was responsible for the observed mosaic phenotype, total RNA and protein were extracted from 12-day-old plants from different lines and used for RNA blot, real time quantitative RT-PCR, and immunoblot analyses. As expected, DXR transcripts in variegated R-3 plants were significantly lower than those in untransformed plants (Fig. 4A, B). No major changes in DXR transcript levels were observed in populations of R-1 plants (Fig. 4A), which included mostly green plants but also a few variegated individuals (Fig. 2C). When analyzed separately, however, a decreased accumulation of DXR transcripts was observed in variegated R-1 individuals compared to green plants of the same line, which showed levels only slightly higher than those in untransformed controls (Fig. 4B). Together, a clear correlation exists between decreased DXR transcript levels and the mosaic phenotype. In the case of the R-2 line, which did not produce variegated individuals, *DXR* was clearly overexpressed (Fig. 4A, B). Immunoblot analysis with an anti-DXR serum (Rodríguez-Concepción et al. 2001; Carretero-Paulet et al. 2002) showed that enzyme levels followed the same pattern of transcript accumulation, with decreased levels in R-3 plants and increased levels in the R-2 line relative to R-1 and untransformed plants (Fig. 4C). The altered levels of DXR in transgenic *35S:DXR* lines did not result in changes in the accumulation of endogenous DXS transcripts or protein (Fig. 4B, C), suggesting that the phenotypes observed in these lines were only due to changes in DXR levels.

Altered DXR or DXS levels result in differential resistance to MEP pathway inhibitors

Immunoblot analysis of DXR accumulation in other *35S:DXR* lines visually undistinguishable from the wild-type showed that only some of them accumulated higher levels of the enzyme compared to

untransformed plants (data not shown). In the absence of available methods to readily measure DXR activity in plant extracts, we studied whether an increase in enzyme activity in transgenic plants could be estimated from their resistance to fosmidomycin (FSM), a specific inhibitor of DXR activity and plastidial isoprenoid biosynthesis in plants (Zeidler et al. 1998; Rodríguez-Concepción et al. 2001; Okada et al. 2002; Laule et al. 2003; Rodríguez-Concepción et al. 2004; Guevara-García et al. 2005). Sublethal concentrations of FSM in the growth medium of wild-type *Arabidopsis* cause a bleached phenotype and a developmental arrest (Fig. 5A, B), whereas concentrations of 50 μM FSM or higher result in an albino phenotype and a complete block in the production of true leaves by the shoot apical meristem of wild-type seedlings (Fig. 5C). To investigate the resistance of the selected *35S:DXR* lines to FSM, seeds were germinated and grown on MS plates supplemented with FSM. The degree of resistance to the inhibitor was quantified as the percentage of seedling establishment (SE), i.e., the proportion of seedlings capable of producing green true leaves and develop relative to the total number of seedlings. As shown in Fig. 6A, five *35S:DXR* lines (R-1, R-3, R-8, R-10, and R-16) did not produce seedlings with green true leaves in the presence of 50 μM FSM. Quantification of SE rates of R-1 and R-3 homozygous populations in 25 μM FSM showed that only the R-3 line was significantly more sensitive to FSM than untransformed seedlings (Fig. 6B). The highest FSM resistance level was observed for R-2 seedlings (Fig. 6A), many of which could develop normally in the presence of the inhibitor (Figs. 5F–H, 6B). These results are consistent with DXR being the target for FSM in plants and suggest that increased resistance to the inhibitor in some *35S:DXR* lines might be a good indicator of higher DXR activity levels. As FSM is a competitive inhibitor in the conversion of DXP to MEP (Steinbacher et al. 2003), increased DXS activity in *35S:DXS* plants was also expected to result in enhanced resistance to FSM. In agreement, S-5 and S-6 seedlings showed FSM resistance (Figs. 5K–M, 6A, B), likely due to a higher production of DXP that competes with the inhibitor.

A number of inhibitors of the MEP pathway besides FSM have been described, but only a few have been studied in plants (Rodríguez-Concepción 2004). Clomazone (CLM), a commonly used herbicide, has been reported to inhibit DXS activity after conversion to an active compound in plants (Zeidler et al. 2000). As shown in Fig. 5, not all the tissues in the *Arabidopsis* plant are equally sensitive to this herbicide. Germination and growth of wild-type seedlings in the presence

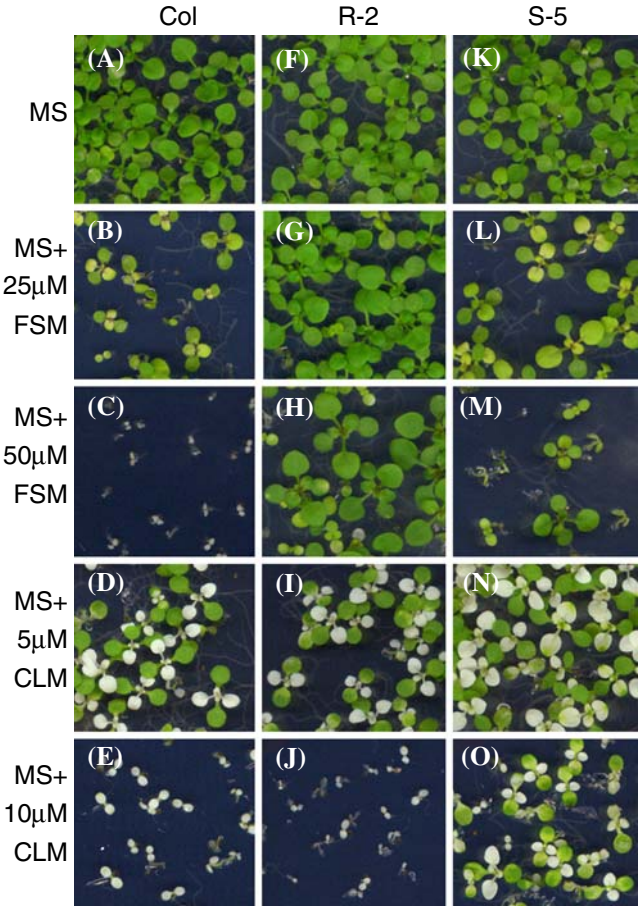

Fig. 5 Resistance of 35S:DXR and 35S:DXS lines to MEP pathway inhibitors. Seeds from untransformed (**A**–**E**), R-2 (**F**–**J**), and S-5 (**K**–**O**) plants were surface-sterilized, germinated on MS plates either supplemented or not with CLM or FSM, and incubated under LD photoperiod for 15 days. First row (pannels **A**, **F**, **K**): non-supplemented medium; second row (**B**, **G**, **L**): 25 μM FSM; third row (**C**, **H**, **M**): 50 μM FSM; fourth row (**D**, **I**, **N**): 5 μM CLM; last row (**E**, **J**, **O**): 10 μM CLM

of low concentrations of CLM results in green cotyledons but albino true leaves (Fig. 5D). Increased concentrations of the inhibitor cause a progressive loss of pigment accumulation in the cotyledons and a concomitant inhibition of true leaf development. At concentrations of 10 μM CLM or higher, the biosynthesis of photosynthetic pigments and the production of true leaves is blocked, resulting in a seedling albino phenotype with a developmental block (Fig. 5E). S-5 and S-6 seedlings were clearly resistant to CLM, in agreement with DXS activity being the target of this inhibitor or a derivative (Figs. 5N, O, 6C). By contrast, seedlings from lines with reduced (R-3) or increased (R-2) DXR levels showed a wild-type sensitivity to CLM (Figs. 5I, J, 6C), confirming that altered DXR levels do not lead to changes in DXS. These results illustrate that DXS or DXR levels in transgenic plants

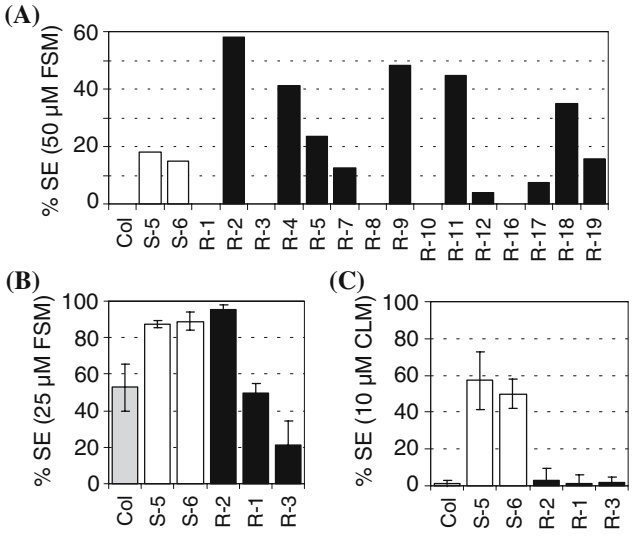

Fig. 6 Quantification of FSM and CLM resistance. Resistance was estimated with populations of more than 50 individuals as the percentage of seedling establishment (SE) after growth for 15 days under LD conditions on MS plates supplemented with the indicated concentrations of FSM or CLM. Values in **(B)** and **(C)** represent the mean and standard deviation from at least three independent experiments

can be indirectly estimated by monitoring resistance to CLM and FSM, respectively.

Increased DXR levels lead to enhanced accumulation of plastid isoprenoids

Overexpression of DXS in *Arabidopsis* has been previously shown to result in increased levels of plastidial isoprenoids such as chlorophylls, carotenoids, and tocopherol (Estévez et al. 2001), suggesting that the production of DXP by DXS limits the synthesis of IPP and DMAPP. To determine whether an enhanced DXR activity could also result in increased isoprenoid production in chloroplasts, the levels of chlorophylls and carotenoids were measured by HPLC in homozygous T_3 populations of seedlings from *35S:DXR* lines showing FSM resistance (R-2, R-9, R-11, and R-18), and from the silenced R-3 line (Fig. 7). The R-1 line, which showed wild-type levels of total DXR accumulation and FSM resistance (Figs. 4, 6), and the DXS-overexpressing lines S-5 and S-6 were also analyzed as controls. As expected, S-5 and S-6 plants showed much increased levels of chlorophylls and carotenoids compared to untransformed plants (Fig. 7A). A moderate (ca. 25%) but significant increase was also observed in all *35S:DXR* lines tested but R-1, which showed wild-type levels of these pigments, and R-3, in which a reduction of ca. 20% was detected (Fig. 7A). These results indicate a correlation between DXR levels and accumulation of plastidial isoprenoids.

To confirm that the observed increase in chlorophyll and carotenoid levels in plants overexpressing DXR was due to the enhanced production of their metabolic precursors, we monitored the production of taxadiene, a non-native plastidial isoprenoid in *Arabidopsis* that is directly synthesized from GGPP (Fig. 1) by the activity of yew (*Taxus baccata*) taxadiene synthase (TXS) in transgenic *35S:TXS* plants (Besumbes et al. 2004). Our earlier work showed that overexpression of the MEP pathway enzymes DXS or HDR in the *35S:TXS* background led to a dramatic increase in the accumulation of taxadiene compared to single *35S:TXS* transgenic plants, confirming that an enhanced production of isoprenoid precursors resulted in higher rates of taxadiene biosynthesis (Botella-Pavía et al. 2004). To estimate whether higher DXR levels could also result in increased flux through the MEP pathway, DXR-overexpressing plants of the R-2 line were crossed with homozygous *35S:TXS* plants. As controls, *35S:TXS* plants were also crossed with *35S:DXS* plants (line S-5) and untransformed plants. At least three F_1 plants from each of the three crosses were allowed to self-fertilize. Seedlings carrying at least one 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 chlorophylls and carotenoids in the selected seedlings. As shown in Fig. 7B, seedlings from the TXS + DXS and TXS + DXR crosses contained higher chlorophyll and carotenoid levels than those derived from the cross with the wild-type, therefore confirming that both *35S:DXS* and *35S:DXR* constructs remained active and that an increased activity of the corresponding proteins results in enhanced production of plastidial isoprenoids. When samples were used to measure taxadiene content by GC-MS, a 6.5-fold and 3.5-fold higher increase in the accumulation of taxadiene was detected in TXS + DXS and TXS + DXR seedlings, respectively, compared to control seedlings carrying only the

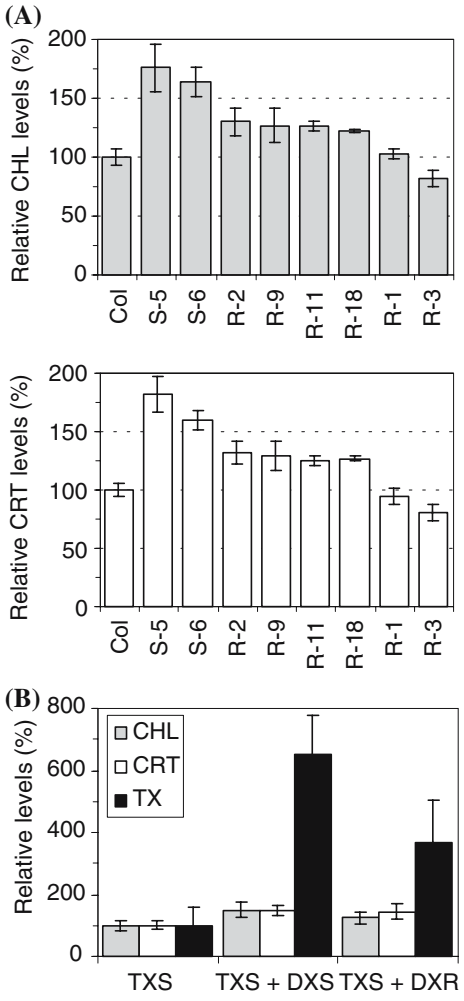

Fig. 7 Levels of plastid isoprenoids in transgenic lines. (**A**) Chlorophyll (CHL) and carotenoid (CRT) contents of transgenic lines relative to those measured in untransformed (Col) plants grown under the same conditions (15 days on MS plates under LD photoperiod). Mean and standard deviation correspond to two independent experiments with two replicas each. (**B**) Chlorophyll, carotenoid, and taxadiene (TX) levels in F_2 seedlings from crosses of homozygous 35S:TXS plants with untransformed (TXS), S-5 (TXS + DXS) or R-2 (TXS + DXR) plants. Two pools of seedlings carrying the parental markers were selected from each population. Mean and standard deviation of two separate extractions from each pool are shown. Levels are represented relative to those in the TXS sample

35S:TXS transgene (Fig. 7B). These results confirm a role for both DXS and DXR in controlling the metabolic flux of the MEP pathway to GGPP and downstream plastidial isoprenoid end-products.

Discussion

The identification of genes involved in the biosynthesis of plastid isoprenoids of commercial value such as monoterpenes, carotenoids, or Taxol has opened the door to their biotechnological overproduction in plants. Since the supply of precursors appears to limit their production (Fray et al. 1995; Lois et al. 2000; Estévez et al. 2001; Mahmoud and Croteau 2001; Okada et al. 2002; Besumbes et al. 2004; Botella-Pavía et al. 2004; Enfissi et al. 2005), metabolic engineering approaches aimed to synthesize high levels of such products should also take into account the requirement to up-regulate the flux through the MEP pathway. By analyzing *Arabidopsis* plants in which DXR levels are either decreased or increased relative to the wild-type, we provide evidence here that the step catalyzed by DXR (the biosynthesis of MEP, the first committed intermediate of the pathway) exerts control over this flux in *Arabidopsis* chloroplasts.

It has been well established that a block of the MEP pathway in mutants and transgene-mediated silenced plants leads to impaired chloroplast differentiation and eventually a pale or albino phenotype (Mandel et al. 1996; Araki et al. 2000; Estévez et al. 2000; Budziszewski et al. 2001; Nagata et al. 2002; Okada et al. 2002; Gutiérrez-Nava et al. 2004; Page et al. 2004; Hsieh and Goodman 2005a, b). Unlike the nearly uniform lack of pigmentation caused by null and intermediate *DXS* alleles such as *cla1* (Mandel et al. 1996; Estévez et al. 2000; Nagata et al. 2002) and *chs5* (Araki et al. 2000), the weak *lvr111* mutant (Crowell et al. 2003) produces pale seedlings and small plants with variegated leaves. Similarly, *DXR* knockout mutants have a seedling lethal, albino phenotype (Budziszewski et al. 2001), whereas a variegated phenotype was observed here in R-3 and some R-1 plants which showed down-regulated DXR transcript levels (Figs. 2, 4). It is therefore likely that the variegated phenotype of these plants is due to a decreased DXR activity, as estimated from the lower resistance of the R-3 line to FSM (Figs. 5, 6). Consistently, transgenic peppermint plants with decreased *DXR* transcript levels showed a mosaic pattern, whereas strongly silenced lines with no detectable DXR activity were uniformly pale (Mahmoud and Croteau 2001). Green patches within areas of white tissue have also been observed in leaves from *Nicotiana benthamiana* plants when virus-mediated silencing of the genes encoding HDS or HDR was not complete (Page et al. 2004). Only line R-3 was used in this work to further analyze the biological effects of DXR down-regulation, basically because the other lines showing variegated individuals only produced them occasionally. However, the fact that very similar mosaic phenotypes are also observed in other plants with reduced levels of DXR and other MEP pathway enzymes supports that the R-3

phenotype is not the result of an unspecific event but most likely caused by a decreased production of MEP and dowstream metabolites. The lower accumulation of chlorophylls and carotenoids in R-3 seedlings (Fig. 7) further supports that a reduced production of MEP results in decreased levels of plastidial isoprenoids, which might eventually affect the development of chloroplasts (Fig. 3). Our results also confirm that, under normal growth conditions, the import of MVA-derived prenyl diphosphates into the chloroplast is not active enough to rescue the production of photosynthetic pigments in *Arabidopsis* seedlings with a genetic (Fig. 2) or pharmacological (Fig. 5) block of the MEP pathway.

Besides demonstrating the specificity of the MEP pathway inhibitors FSM and CLM towards DXR and DXS, respectively (Figs. 5, 6), we showed that increased levels of any of these enzymes lead to a higher accumulation of primary isoprenoids such as chlorophylls and carotenoids (Fig. 7). A relevant contribution of our work is the demonstration that the changes in DXR levels in transgenic plants do not lead to changes in DXS enzyme accumulation (Fig. 4) or activity (as estimated from CLM resistance; Figs. 5, 6), confirming that the observed effects on chloroplast isoprenoid levels in *35S:DXR* lines (Fig. 7) are not an indirect consequence of altering DXS levels. Although different levels of transcript, protein, and DXR activity (as estimated from FSM resistance) were observed in *35S:DXR* plants, the increase in the production of chlorophylls and carotenoids was very similar in all the DXR-overexpressing lines tested (ca. 25% higher than that in untransformed plants). These results suggest that the levels of isoprenoid pigments cannot be increased beyond that point by up-regulating DXR in chloroplasts. The fact that DXS-overexpressing plants grown under the same conditions accumulated higher levels of isoprenoid pigments than *35S:DXR* plants (Fig. 7) suggests that the production of DXP might become limiting when DXR activity is increased. The production of taxadiene, a non-native plastidial isoprenoid in *Arabidopsis* directly formed from GGPP by a recombinant TXS enzyme from *Taxus baccata* (Fig. 1), was also boosted in plants overexpressing DXS or DXR. The increase in taxadiene accumulation was much higher than the observed raise in chlorophyll and carotenoid levels (Fig. 7). A possible reason is that recombinant TXS (whose expression is driven by the strong constitutive *35S* promoter) can be produced at much higher levels than the endogenous chlorophyll and carotenoid biosynthetic enzymes. If taxadiene production is limited by precursor availability, a substantial amount of the extra GGPP formed in TXS + DXS and TXS + DXR double transgenic plants might be used by excess TXS to readily synthesize taxadiene, whereas lower levels of MEP-derived precursors would be used for photosynthetic pigments. The diversion of the additional precursor molecules to complex branched pathways for chlorophyll and carotenoid biosynthesis might also contribute to explain the observed differential increase in the levels of these compounds compared to taxadiene. Furthermore, the levels of taxadiene are more directly dependent on the supply of MEP-derived precursors (Besumbes et al. 2004), whereas additional regulatory steps in the downstream pathways leading to chlorophyll and carotenoid biosynthesis are known to limit the accumulation of these isoprenoid pigments after up-regulation of the MEP pathway (Estévez et al. 2001; Enfissi et al. 2005). In any case, taxadiene production might be a good estimate of the availability of isoprenoid precursors in transgenic plants. Similar to that observed for chlorophylls and carotenoids, plants with up-regulated DXS levels showed a higher increase in taxadiene accumulation than DXR-overexpressing plants, consistent with a prominent role of DXS in controlling the production of IPP and DMAPP in chloroplasts. Together, these results support the metabolic control analysis (MCA) conclusion that several enzymes can share control over the flux of the MEP pathway, with different enzymes exhibiting different degrees of control (Fell 1992; Thomas and Fell 1998). This is in contrast with the MVA pathway, in which only overexpression of hydroxymethylglutaryl coenzyme A reductase (HMGR), the enzyme catalyzing the first committed step of the pathway, results in increased levels of sterols, the main group of MVA-derived isoprenoids (Gondet et al. 1992; Chappell et al. 1995; Schaller et al. 1995; Harker et al. 2003; Enfissi et al. 2005; Manzano et al. 2004). These results suggest that HMGR catalyzes the key regulatory step of the MVA pathway.

Both DXS and DXR, which channel two central plant metabolites such as pyruvate and GAP to the committed production of isoprenoids in plastids, were considered potential regulators of flux through the MEP pathway soon after their discovery. Several lines of evidence, including overexpression studies in *Arabidopsis* and tomato (Estévez et al. 2001; Enfissi et al. 2005), led to conclude that the step catalyzed by DXS was indeed limiting for the biosynthesis of plastidial isoprenoids in plant cells. However, the relative contribution of DXR to the supply of precursors remained unclear. Unlike that shown for DXS, DXR levels do not correlate with the accumulation of carotenoids in the chromoplasts of ripening tomato fruit (Rodríguez-Concepción et al. 2001) or the diurnal

oscillation of the biosynthesis and emission of MEP-derived isoprenoid volatiles in snapdragon flowers (Dudareva et al. 2005). By contrast, a positive correlation was found between enhanced isoprenoid biosynthesis and accumulation of transcripts encoding DXR in other systems (Walter et al. 2000; Veau et al. 2000; Carretero-Paulet et al. 2002; Hans et al. 2004; Hsieh and Goodman 2005a; Mayrhofer et al. 2005; Bede et al. 2006). Furthermore, transgene-mediated alteration of DXR levels in peppermint plants led to concomitant changes in the production of monoterpene essential oils in leukoplasts (Mahmoud and Croteau 2001). The results reported here reveal that the production of MEP from DXP catalyzed by DXR also limits the biosynthesis of isoprenoids in chloroplasts. In conclusion, our work has contributed to establish that regulation of IPP and DMAPP synthesis in *Arabidopsis* chloroplasts is shared by DXR and at least two other MEP pathway enzymes, DXS and HDR (Botella-Pavía et al. 2004). Other transgenic approaches would be required to determine whether the regulatory nature shown for DXR is restricted to certain plants, tissues, developmental stages, or even plastids types.

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