Plastid Cues Posttranscriptionally Regulate the Accumulation of Key Enzymes of the Methylerythritol Phosphate Pathway in Arabidopsis[1]

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Plastid isoprenoids (including hormones and photosynthetic pigments) are essential for plant growth and development, but relatively little is known of how the production of their metabolic precursors via the recently elucidated methylerythritol phosphate (MEP) pathway is regulated. We have identified an Arabidopsis (*Arabidopsis thaliana*) mutant that survives an otherwise lethal block of the MEP pathway with fosmidomycin (FSM). In *rif10* (*resistant to inhibition with FSM 10*) plants, the accumulation of flux-controlling enzymes of the pathway is posttranscriptionally up-regulated. Strikingly, this phenotype is linked to a lower accumulation of plastidial isoprenoid pigments such as chlorophylls and carotenoids, resulting in mutant plants that are paler and smaller than the wild type. The *rif10* mutant is impaired in plastid RNA processing due to a T-DNA insertion in the coding region of the At3g03710 gene encoding the chloroplast-targeted exoribonuclease polyribonucleotide phosphorylase. FSM resistance and other *rif10*-like phenotypes were also observed in wild-type Arabidopsis, tomato (*Lycopersicon esculentum*), and rice (*Oryza sativa*) seedlings grown in the presence of sublethal concentrations of chloramphenicol (an inhibitor of protein synthesis in plastids). By contrast, treatment with norflurazon (an inhibitor of carotenoid biosynthesis causing a similar pale cotyledon phenotype) did not result in FSM resistance. Together, the results support that plastome-encoded proteins are involved in negatively regulating the posttranscriptional accumulation of specific nuclear-encoded MEP pathway enzymes in chloroplasts. Regulation of the MEP pathway by a mechanism dependent on plastid cues might function under physiological conditions to finely adjust plastidial isoprenoid biosynthesis to the metabolic capabilities or requirements of plastids.

Isoprenoids are an extremely diverse group of compounds synthesized by all organisms but particularly abundant and diverse in plants (Chappell, 1995; Croteau et al., 2000). All isoprenoids derive from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Condensation of IPP and DMAPP units leads to the synthesis of prenyl diphosphates of increasing size that are the starting points for multiple branches leading to the final isoprenoid products. Unlike most organisms, plants use two pathways to synthesize IPP and DMAPP in different compartments (Lichtenthaler, 1999; Eisenreich et al., 2001; Rodríguez-Concepción and Boronat, 2002). The mevalonic acid pathway produces cytosolic IPP for sterols, brassinosteroids, triterpenes, sesquiterpenes, polyterpenes, dolichol, and the isoprenyl groups used for protein prenylation and cytokinin biosynthesis. The side chain of ubiquinones is also formed from mevalonic acid-derived IPP synthesized in the cytosol and imported into the mitochondria. On the other hand, most plant isoprenoids, including photosynthesis-related compounds (carotenoids and the side chain of chlorophylls, tocopherols, phylloquinones, and plastoquinones), hormones (cytokinins, gibberellins, and abscisic acid), isoprene, and monoterpenes, derive from precursors synthesized in plastids by the recently elucidated methylerythritol phosphate (MEP) pathway (Fig. 1A). Despite compartmentation of the biosynthesis of isoprenoid precursors, a limited exchange of IPP or a downstream prenyl diphosphate has been shown to take place between the cytosol and the plastid in at least some plants, including Arabidopsis

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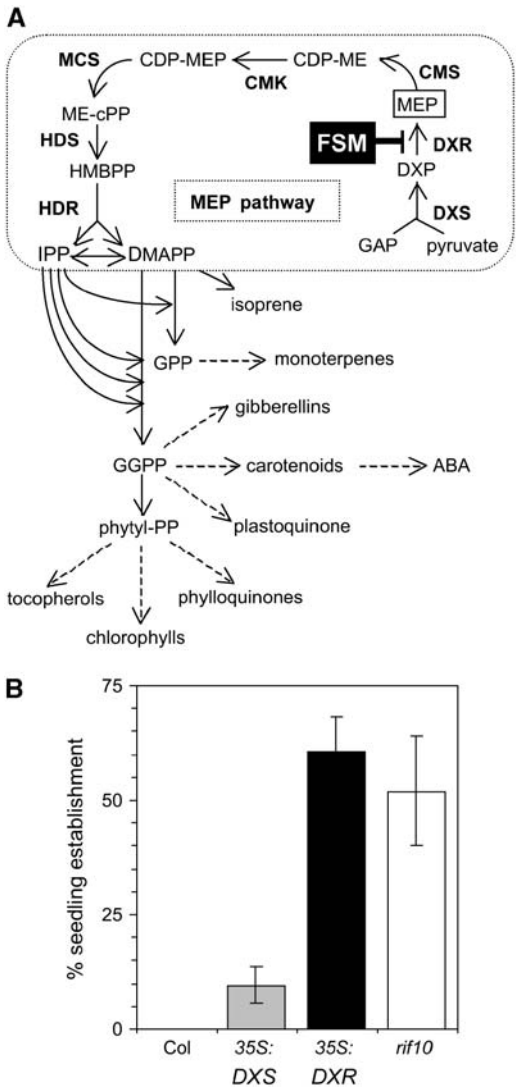

Figure 1. Plastidial isoprenoids and FSM resistance. A, Schematic pathway for the biosynthesis of MEP-derived isoprenoids. Multiple steps are indicated with striped arrows. The step inhibited by FSM is shown. GAP, glyceraldehyde 3-P; DXP, deoxyxylulose 5-P; MEP, methylerythritol 4-P; CDP-ME, 4-diphosphocytidyl-methylerythritol; CDP-MEP, CDP-ME 2-P; ME-cPP, methylerythritol 2,4-cyclodiphosphate; HMBPP, hydroxymethylbutenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; ABA, abscisic acid. Enzymes are indicated in bold. DXS, DXP synthase; DXR, DXP reductoisomerase; CMS, CDP-ME synthase; CMK, CDP-ME kinase; MCS, ME-cPP synthase; HDS, HMBPP synthase; HDR, HMBPP reductase. B, Quantification of FSM resistance of seedlings from the Col wild-type, transgenic plants constitutively overexpressing either DXS (*35S:DXS*) or DXR (*35S:DXR*), and the *rif10* mutant. Resistance was measured as the percentage of seedlings that developed green true leaves (SE) as visually determined after growth for 14 d under LD conditions on MS plates supplemented with 50 μM FSM. Values represent the mean and SD from populations of more than 50 individuals in several independent experiments.

(*Arabidopsis thaliana*; Kasahara et al., 2002; Nagata et al., 2002; Laule et al., 2003). In light-grown Arabidopsis seedlings, however, the exchange rate is not high enough to rescue a block of one of the two pathways with common isoprenoid precursors synthesized by the other pathway (Estévez et al., 2000; Budziszewski et al., 2001; Gutiérrez-Nava et al., 2004; Rodríguez-Concepción et al., 2004; Suzuki et al., 2004). Therefore, pathway-specific mechanisms must exist to ensure that isoprenoid precursors will be produced in each compartment when needed.

It is now well established that all the MEP pathway enzymes are encoded by nuclear genes and imported into plastids (Rodríguez-Concepción and Boronat, 2002; Eisenreich et al., 2004). The initial reaction of the MEP pathway, catalyzed by deoxyxylulose 5-P (DXP) synthase (DXS), involves the production of DXP from glyceraldehyde 3-P and pyruvate. In the second step, the enzyme DXP reductoisomerase (DXR) transforms DXP into MEP, currently considered the first committed precursor of plastid isoprenoids. MEP production can be blocked by fosmidomycin (FSM), a strong inhibitor of DXR (Steinbacher et al., 2003). FSM causes a bleached phenotype and a block in the production of true leaves by the shoot apical meristem, eventually resulting in a seedling-lethal phenotype (Laule et al., 2003; Rodríguez-Concepción et al., 2004). After conversion of MEP into methylerythritol 2,4-cyclodiphosphate in three enzymatic steps, a reduction catalyzed by hydroxymethylbutenyl diphosphate (HMBPP) synthase (HDS) produces HMBPP, which is finally converted by the enzyme HMBPP reductase (HDR) into IPP and DMAPP (Fig. 1A). In contrast with the impressive progress in the elucidation of the MEP pathway, relatively little is currently known on the regulatory mechanisms that modulate the metabolic flux through the pathway. Besides the control exerted by changes in the expression of genes encoding the biosynthetic enzymes in response to developmental, environmental, and metabolic signals (for review, see Rodríguez-Concepción, 2006), it has been proposed that enzyme levels might be regulated at translational or posttranslational levels in response to developmental cues and changes in the MEP pathway flux (Guevara-García et al., 2005). The impact of these changes on enzyme activity, however, was not evaluated, and therefore their biological relevance remains to be established.

To get a deeper insight into how plants control the production of isoprenoid precursors in the plastid, we searched for Arabidopsis mutants able to survive a block of the MEP pathway with FSM. Here, we report the isolation and characterization of one such FSM-resistant mutant, *rif10* (*resistant to inhibition with FSM 10*), in which a loss of function of the chloroplast-targeted exoribonuclease polyribonucleotide phosphorylase (PNPase) unexpectedly resulted in the posttranscriptional up-regulation of DXR and three other enzymes of the MEP pathway (DXS, HDS, and HDR). Efficient RNA processing (including endonucleolytic cleavage of polycistronic transcripts and

exonucleolytic 5′ and 3′ end maturation of precursor RNAs) is key for the correct expression of the plastid genome, as unprocessed or incorrectly processed RNAs are subject to rapid degradation by ribonucleases (Sugita and Sugiura, 1996; Monde et al., 2000). Transgene-mediated alteration of PNPase activity in Arabidopsis chloroplasts has shown that this enzyme is required for efficient 3′ end processing of plastome-encoded mRNAs and a 23S rRNA precursor. In addition, PNPase participates in the regulation of tRNA turnover and polyadenilated transcript degradation (Walter et al., 2002). Impaired metabolism of plastid RNAs in *rif10* seedlings leads to decreased levels of plastome-encoded proteins, which in turn results in lower levels of chlorophylls and carotenoids despite the up-regulation of MEP pathway enzyme levels. While the essential contribution of MEP-derived isoprenoids for plastid differentiation has been well established from the characterization of mutants defective in the MEP pathway (Mandel et al., 1996; Nagata et al., 2002; Gutiérrez-Nava et al., 2004), our results provide evidence that plastid signals can in turn modulate the accumulation in chloroplasts of nuclear-encoded MEP pathway enzymes with a potential regulatory role for the production of plastidial isoprenoid precursors.

RESULTS

Identification of FSM-Resistant Mutants

To get new insights into the regulation of the MEP pathway in plants, we aimed to identify mutants that could resist the presence of concentrations of FSM that are lethal for the Columbia (Col) wild-type. The minimum concentration of inhibitor at which all Arabidopsis wild-type seedlings showed an albino phenotype and a complete developmental arrest was 50 μM FSM (Figs. 1B and 2A). FSM resistance was visually estimated from the rescue of the bleached phenotype and quantified as the percentage of seedling establishment (SE), i.e. the proportion of seedlings developing green true leaves that can support further plant development. As shown in Figure 1B, more than one-half of the seedlings from *35S:DXR* transgenic lines overexpressing DXR under the control of the constitutive cauliflower mosaic virus *35S* promoter produce green cotyledons and true leaves and develop normally in the presence of the inhibitor, consistent with DXR being the specific target of FSM. Because FSM is a competitive inhibitor of DXR (Steinbacher et al., 2003), an increase in the levels of its substrate (DXP) should also result in resistance to the inhibitor. In agreement, transgenic *35S:DXS* plants constitutively overexpressing the Arabidopsis DXS enzyme also show resistance to FSM, although at much lower levels than *35S:DXR* plants, as estimated from their SE rates in the presence of 50 μM FSM (Fig. 1B). The fact that only some individuals in homozygous populations of transgenic *35S:DXS* or *35S:DXR* lines can survive in the presence of FSM suggests the existence of epigenetic factors modulating the resistance to the inhibitor. However, our results validate the use of FSM to specifically inhibit the production of MEP-derived isoprenoids in Arabidopsis and to screen for mutants with an up-regulated MEP pathway.

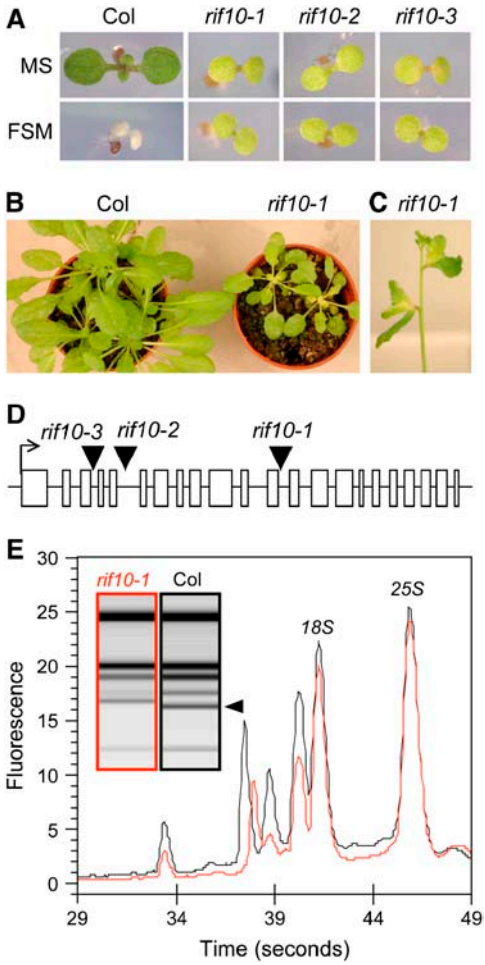

Figure 2. Characterization of the *rif10* mutant. A, Representative wild-type (Col) and homozygous seedlings of three different *rif10* alleles (see D) germinated on MS plates either supplemented or not with 50 μM FSM and grown under LD conditions for 6 d. Sections are to the same scale. B, Col and *rif10-1* plants grown on soil for 25 d under LD conditions. C, Mutant *rif10-1* inflorescence. D, Map of the *RIF10* gene (At3g47450) encoding plastid PNPase. The translation start (arrow) and the exons (boxes) are indicated. The position of the T-DNA in *rif10-1*, *rif10-2*, and *rif10-3* mutants is also represented. E, Bioanalyzer electropherogram and gel-like image (inset) of total RNA from 10-d-old Col and *rif10-1* seedlings. Arrowhead indicates the 23S rRNA fragment shifted in size in the mutant.

For the identification of FSM-resistant mutants, seeds from public collections of T-DNA insertion lines generated with a construct for activation tagging (Weigel et al., 2000) were germinated on Murashige and Skoog (MS) plates supplemented with 50 μM FSM. Seedlings that produced at least two sets of green true leaves were transferred to soil and allowed to develop and set seed. The lines showing a consistent FSM-resistance phenotype in the next generation were named

rif. One of the selected mutants, *rif10*, showed a FSM resistance phenotype similar to that observed in Arabidopsis plants overexpressing DXR (Fig. 1B). This line was therefore selected for further characterization.

Mutant *rif10* Seedlings Show a Delayed Greening and Growth Phenotype Linked to FSM Resistance

During the first days following germination on 50 μM FSM, *rif10* seedlings were largely unaffected by the inhibitor (Fig. 2A). On FSM-free medium, however, *rif10* seedlings required more time than the wild type for both greening and development of true leaves (Fig. 2A). The slower growth rate of the mutant resulted in *rif10* plants that were much smaller than Col plants grown under the same conditions (Fig. 2B). Adult *rif10* plants also displayed a characteristic virescent phenotype, i.e. young leaves and recently expanded tissues (including the basal area of older leaves and young inflorescence shoots) were pale, whereas more mature tissues were as green as in the wild type (Fig. 2, B and C). Despite the described phenotypic alterations, *rif10* plants were viable and fertile under our normal growth conditions.

For the identification of the gene mutated in *rif10*, homozygous mutant plants were backcrossed with the Col wild type and the following generations were studied. Genetic analyses (data not shown) showed that FSM resistance and the slow greening and growth phenotypes were all recessive and linked to the presence of the T-DNA used to generate the lines (Weigel et al., 2000). Analysis of the T-DNA flanking sequences in the *rif10* genome showed that the T-DNA insertion interrupted the coding region of the At3g03710 gene. Other insertion alleles identified in the Salk collection (Salk_037353 and Salk_013306) were referred to as *rif10-2* and *rif10-3*, respectively, after renaming *rif10* as *rif10-1* (Fig. 2D). Homozygous *rif10-2* and *rif10-3* plants displayed all the distinctive phenotypes reported for *rif10-1*, including a developmental delay, paler green cotyledons, and FSM resistance (Fig. 2A). These results confirmed that the described phenotypes were caused by the loss of function of the At3g03710 (*RIF10*) gene. *RIF10* encodes PNPase, a plastid-targeted exoribonuclease implicated in the metabolism of all major classes of plastid RNAs, including mRNAs, tRNAs, and the 23S rRNA (Walter et al., 2002). One of the most obvious molecular phenotypes displayed by plants with a transgene-induced reduction of PNPase activity was the reduced mobility of a plastid 23S rRNA precursor due to the defective exonucleolytic trimming of 98 nucleotides of its 3′ end (Walter et al., 2002). The mobility shift was also observed in *rif10* seedlings (Fig. 2E), confirming a loss of PNPase activity in the mutant.

FSM Resistance of *rif10* Seedlings Can Be Explained by a Posttranscriptional Up-Regulation of DXS and DXR Levels

The most direct mechanism for FSM resistance is the up-regulation of DXR or even DXS levels (Fig. 1). To investigate whether the resistance of *rif10-1* seedlings to FSM resulted from an increased accumulation of any of these MEP pathway enzymes, we compared transcript and protein levels in wild-type and mutant seedlings. RNA-blot analysis with gene-specific probes showed that similar *DXS* and *DXR* transcript levels were present in Col and mutant seedlings (Fig. 3A). However, immunoblot analysis with antibodies raised against the Arabidopsis DXS and DXR proteins (Estévez et al., 2000; Rodríguez-Concepción et al., 2001) showed clearly higher amounts of both MEP pathway enzymes in *rif10-1* seedlings (Fig. 3B). The observed phenotype of FSM resistance might therefore derive from the enhanced accumulation of both DXS and DXR enzymes, which might act synergistically. Immunoblot analyses with specific antisera against the rest of the MEP pathway enzymes (Guevara-García et al., 2005) showed that higher amounts of HDS and HDR were present in mutant *rif10-1* seedlings, whereas no major changes were observed in the levels of the rest of the enzymes (Fig. 3C). As described for DXS and DXR, transcript levels encoding HDS and HDR were similar in wild-type and mutant seedlings grown under the same conditions (Fig. 3A). In contrast with the higher accumulation of MEP pathway enzymes, an approximately 50% decrease in the levels of the plastome-encoded Rubisco large subunit (RBCL) was observed in *rif10-1* protein extracts relative to Col (Fig. 3B). Such decrease is consistent with the defects on plastid RNA metabolism and with the pale phenotype of the *rif10* mutants. Together, the results suggest that a defective metabolism of RNAs in mutant chloroplasts might result in decreased synthesis of plastome-encoded proteins such as RBCL, eventually leading to the post-transcriptional accumulation of MEP pathway enzymes.

Increased levels of flux-controlling MEP pathway enzymes such as DXS and HDR might be expected to result in enhanced production of chloroplast isoprenoids such as chlorophylls and carotenoids (Estévez et al., 2001; Botella-Pavía et al., 2004). However, the levels of photosynthetic pigments are not increased, but even decreased, in the mutant (Fig. 3D), consistent with the pale phenotype of *rif10* seedlings (Fig. 2). To confirm whether the up-regulated MEP pathway enzymes were actually active in defective *rif10* chloroplasts, FSM resistance was evaluated in mutant plants in which DXR levels were further up-regulated by transgene-mediated overexpression. Mutant *rif10-1* and transgenic *35S:DXR* plants were crossed, and homozygous *rif10-1* and *rif10-1 35S:DXR* siblings were identified from the analysis of segregating F_3 populations based on resistance marker genes associated with the *rif10-1* mutation and the *35S:DXR* construct. As shown in Figure 4A, *rif10-1 35S:DXR* plants were visually identical to mutant plants lacking the *35S:DXR* transgene. Seedlings from both lines were also indistinguishable, displaying a similar degree of delayed greening and growth and decreased levels of chlorophylls and carotenoids relative to the Col wild type (Fig. 4B). However, *rif10-1 35S:DXR* plants

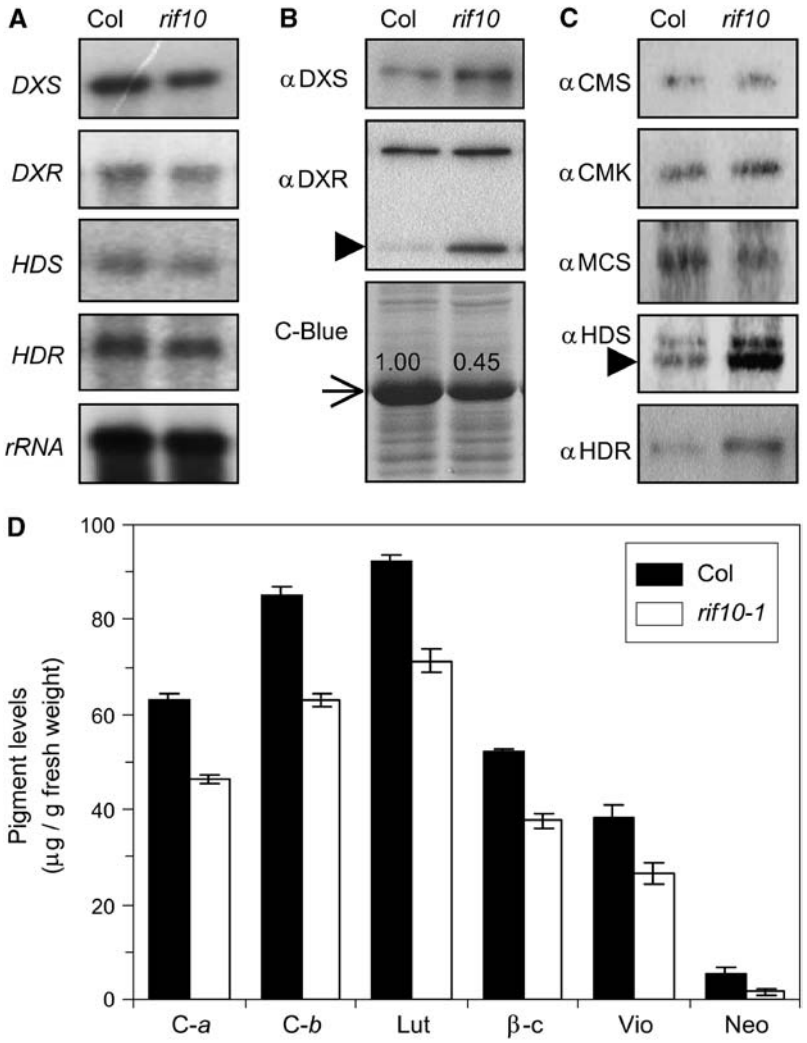

Figure 3. Molecular and biochemical phenotype of *rif10-1* seedlings. RNA, protein, and isoprenoid pigments were extracted from 5-d-old Col and *rif10-1* seedlings grown on MS plates under LD conditions. A, RNA-blot analysis with gene-specific probes. A *25S* rDNA probe was used to compare the RNA amounts loaded in each lane. B, Immunoblot analysis with antibodies raised against DXS or DXR. The position of the DXR protein is indicated with an arrowhead. The other major band recognized by the αDXR serum is shown as a protein loading control. Coomassie Blue (C-Blue) staining was also used to monitor total protein loading. Arrow marks the position of the RBCL protein and numbers represent its relative levels. C, Immunoblot analyses of the same protein extracts with antibodies against the rest of the MEP pathway enzymes. The position of the HDS protein is indicated with an arrowhead. D, Quantification of chlorophylls and carotenoids by HPLC. The mean and SD values from populations of more than 25 seedlings in at least three independent experiments are represented. C-*a*, chlorophyll *a*; C-*b*, chlorophyll *b*; Lut, lutein; β-c, β-carotene; Vio, violaxanthin; Neo, neoxanthin.

showed a significantly higher resistance to FSM (Fig. 4C) and increased DXR levels (Fig. 4D). No changes in DXS levels were observed in *rif10-1 35S:DXR* plants compared to those in the *rif10-1* mutant (Fig. 4D). This, together with the good correlation between DXR protein levels and SE rates in the presence of FSM, indicates that the increased FSM resistance of *rif10-1 35S:DXR* relative to *rif10-1* seedlings was solely caused by an enhanced accumulation of DXR. The results confirm that DXR accumulates in an enzymatically active form in mutant plastids and that the increase in enzyme levels (and not any unspecific effect derived from an altered plastid function) is responsible for the FSM resistance phenotype of *rif10* plants. They also show that factors other than the supply of MEP-derived precursors limit the accumulation of chlorophylls and carotenoids in mutant seedlings.

Pharmacological Inhibition of Plastome Expression Results in a *rif10*-Like Phenotype

To ascertain whether the phenotypes described for mutant *rif10* seedlings (including FSM resistance) were caused by a defective expression of the plastome, we inhibited protein synthesis in wild-type plastids with chloramphenicol (CAP). Indeed, germination and growth of Arabidopsis Col seedlings in the presence of sublethal concentrations of CAP resulted in a *rif10*-like phenotype of pale green cotyledons, delayed development of true leaves, and FSM resistance (Fig. 5A). Similarly, the albino phenotype caused by the inhibition of the MEP pathway with FSM could be partially rescued in tomato (*Lycopersicon esculentum*) and rice (*Oryza sativa*) seedlings when plastid protein synthesis was partially inhibited with CAP (Fig. 5A). The inhibition of the carotenoid biosynthesis pathway in Arabidopsis wild-type seedlings with sublethal concentrations of the herbicide norflurazon (NFZ) resulted in a phenotype very similar to that observed in *rif10* and CAP-treated wild-type seedlings, but it had no effect on FSM resistance (Fig. 5B). These results suggest that the FSM resistance phenotype is not a secondary consequence of impaired plastid development and delayed greening and growth, but most likely a specific effect triggered by decreased plastid protein levels (down-regulated plastome expression).

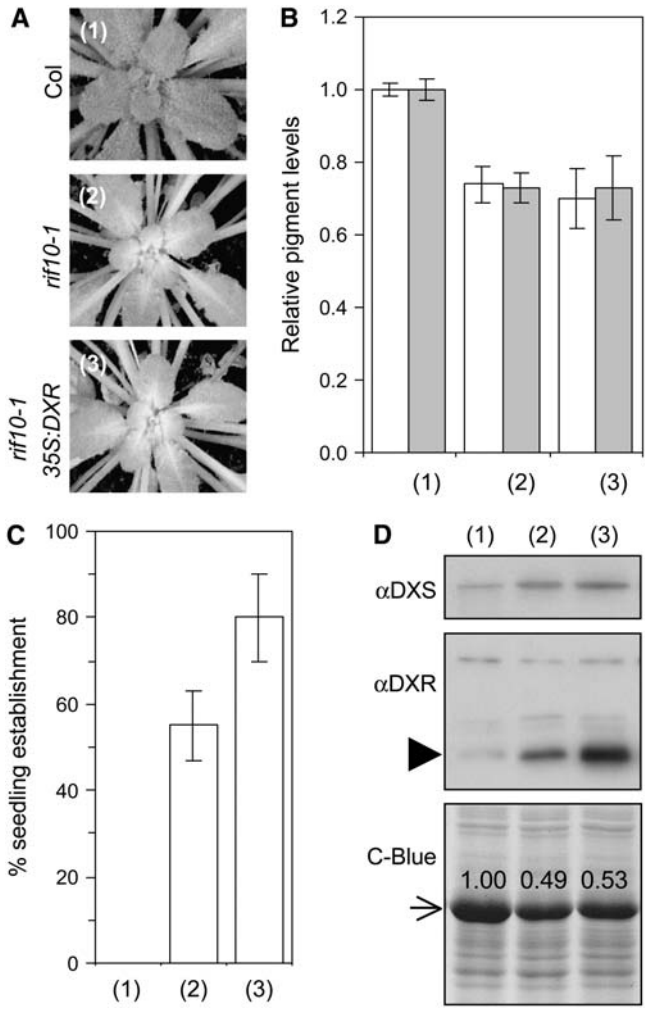

Figure 4. Transgene-mediated overexpression of DXR in *rif10-1* plants. After crossing mutant *rif10-1* and transgenic *35S:DXR* plants, homozygous Col wild-type (1), *rif10-1* (2), and *rif10-1 35S:DXR* (3) siblings were identified based on resistance marker genes associated with the *rif10-1* mutation and the *35S:DXR* construct. A, Young rosette leaves of representative plants. B, Levels of chlorophylls (white columns) and carotenoids (gray columns) relative to those in Col plants. The mean and SD values from populations of more than 25 seedlings in three replicas of two independent experiments are represented. C, Quantification of FSM resistance as the percentage of SE after growth for 14 d under LD conditions on MS plates supplemented with 50 μM FSM. D, Immunoblot analysis of DXS and DXR levels. Arrowhead indicates the position of the DXR protein. Coomassie Blue (C-Blue) staining is also shown. RBCL position and relative levels are indicated.

As expected, lower levels of plastid-encoded proteins such as RBCL were present in CAP-treated Col seedlings (Fig. 6). Also similarly to that described for *rif10-1* seedlings, immunoblot analyses confirmed that the MEP pathway enzymes DXS, DXR, HDS, and HDR accumulated at higher levels in CAP-treated Col seedlings (Fig. 6). A control experiment using a transgenic line constitutively overexpressing the green fluorescent protein (*35S:GFP*) showed that the CAP treatment had no effect on the accumulation of nuclear-encoded proteins that are not targeted to the plastid (Fig. 6). These data confirm that decreased protein synthesis in plastids specifically leads to the posttranscriptional upregulation of four of the seven MEP pathway enzymes, eventually resulting in FSM resistance.

DISCUSSION

Despite the key importance of plastidial isoprenoids for plant life, relatively little is currently known about the regulatory mechanisms that control their production. In this work, we provide genetic and pharmacological evidence that plastids can posttranscriptionally modulate the levels of the nuclear-encoded plastidial MEP pathway enzymes that synthesize their precursors. The loss of function of RIF10/PNPase results in a delayed greening and growth phenotype (Fig. 2) linked to enhanced accumulation of MEP pathway enzymes (Fig. 3) and FSM resistance (Fig. 1B). Transgenic lines with reduced PNPase activity have been shown to accumulate higher levels of unprocessed 23S rRNA precursors, tRNAs, and polyadenylated transcripts susceptible for rapid decay (Walter et al., 2002). Despite additionally showing a reduced accumulation of mature mRNAs encoding RBCL and other photosynthesis-related proteins, PNPase-deficient plants were reported to exhibit no obvious phenotype under various growth conditions and no changes in the levels of RBCL protein (Walter et al., 2002). By contrast, the three *rif10* mutant alleles described here showed a very characteristic phenotype of pale seedlings and virescent plants (Fig. 2), as well as clearly decreased RBCL levels (Figs. 3 and 4), supporting that the complete loss of PNPase activity in mutant plants does lead to decreased levels of chloroplast proteins. The 98 nucleotide extended

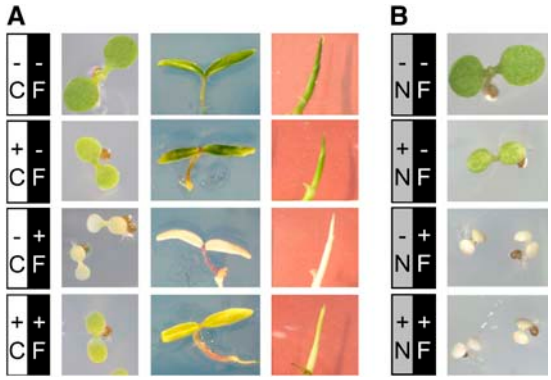

Figure 5. Inhibition of plastid development and derived phenotypes in wild-type seedlings. A, Representative 5-d-old wild-type (Col) Arabidopsis seedlings (left) grown on MS plates supplemented (+) or not (−) with 15 μM CAP (white boxes) and 50 μM FSM (black boxes). Tomato (central) and rice (right) seedlings were grown for 5 d on solid MS medium supplemented or not with 60 μM CAP and 200 μM FSM. B, Representative 5-d-old Col seedlings grown on MS plates supplemented (+) or not (−) with 60 nM NFZ (gray boxes) and 50 μM FSM (black boxes). Seedlings of the same species are shown to the same scale.

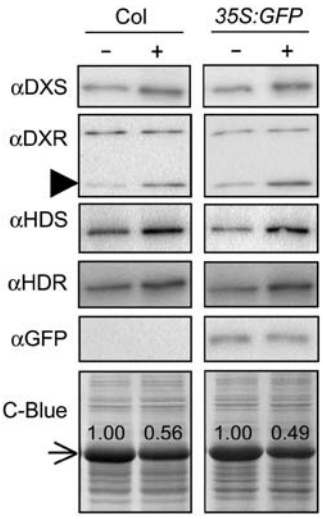

Figure 6. Protein levels in CAP-treated seedlings. Immunoblot analyses with antibodies against the indicated MEP pathway enzymes and against GFP were carried out with protein extracts from 5-d-old Col and *35S:GFP* seedlings grown on MS plates supplemented (+) or not (−) with 15 μM CAP. Arrowhead marks the position of the DXR protein, and the position and relative levels of RBCL are indicated with an arrow and numbers.

23S rRNA precursor detected to be present in *rif10-1* plants can be effectively incorporated into ribosomes, whereas no other rRNA appears to be processed by PNPase (Walter et al., 2002). Therefore, the decreased production of plastome-encoded proteins in the mutant might not be due to impaired plastid ribosome assembly but most likely to enhanced defects in general plastid RNA processing and degradation. A decreased production of plastid proteins in *rif10* plants explains the lower accumulation of chlorophylls and carotenoids in the mutant despite the up-regulated levels of MEP pathway enzymes (Figs. 3 and 4), since both plastome-encoded and nuclear-encoded proteins are required to form the pigment-protein complexes of photosystems that accommodate these photosynthetic pigments in the thylakoid membranes of chloroplasts (Demmig-Adams et al., 1996; Green and Durnford, 1996). Furthermore, the decreased production of plastome-encoded proteins and photosynthetic complexes in *rif10* is expected to result in a lower photosynthetic capacity, which could also explain the delayed growth phenotype of mutant plants (Demmig-Adams et al., 1996; Green and Durnford, 1996; Pesaresi et al., 2001).

The FSM resistance phenotype of *rif10* and CAP-treated wild-type seedlings is likely caused by an enhanced and combined accumulation of DXS and DXR activities in their plastids. Developmental cues have been proposed to be responsible for the post-transcriptional accumulation of high levels of DXS (but not other MEP pathway enzymes) in the early stages of Arabidopsis seedling development (Guevara-García et al., 2005). Although such cues might explain the increased levels of DXS in mutant and CAP-treated seedlings with a developmental delay relative to the wild type (Figs. 2 and 5), the posttranscriptional up-regulation of DXR, HDS, and HDR (Figs. 3 and 6) must be caused by different signals. In the same work, DXS accumulation was also reported to be posttranscriptionally up-regulated in wild-type plants following inhibition of the MEP pathway with FSM, whereas both DXS and HDR levels were shown to be constitutively up-regulated in null mutants of the MEP pathway, suggesting a feedback mechanism in which the levels or ratios of MEP pathway intermediates or products influence the posttranscriptional accumulation of at least some of the pathway enzymes (Guevara-García et al., 2005). Because only processes related to the production of plastome-encoded proteins are disrupted in *rif10* and CAP-treated Col plants, we propose that the observed posttranscriptional accumulation of enhanced levels of the MEP pathway enzymes DXS, DXR, HDS, and HDR is not a consequence of a general arrest in development but most likely a specific response linked to decreased levels of plastome-encoded proteins. In agreement, not all defects in greening or plastid development result in enhanced levels of MEP pathway enzymes (Rodríguez-Concepción et al., 2004; Guevara-García et al., 2005). Most significantly, FSM resistance was not affected in NFZ-treated seedlings, in which the inhibition of carotenoid biosynthesis results in photooxidative damage of chloroplast photosynthetic complexes and a derived phenotype of delayed greening and development very similar to that observed in CAP-treated seedlings (Fig. 5B).

The interest of our results is highlighted by the fact that an involvement of plastome-encoded proteins in the regulation of the MEP pathway was not predicted or expected. Ours, however, is not the first observation of posttranscriptional accumulation of nuclear-encoded plastidial proteins caused by a down-regulation of plastome-encoded proteins. For instance, deletion of the plastid *psbA* gene in tobacco (*Nicotiana tabacum*) triggered a significant up-regulation of plastid terminal oxidase without increasing the levels of transcripts (Baena-González et al., 2003). The mechanism by which plastid-encoded proteins directly or indirectly affect the accumulation of certain plastid-targeted proteins remains unknown. Such a mechanism might act prior to, at, or after the import of the target proteins into the plastid. It has been shown that polyribosome binding, stability, and translation of transcripts of specific nuclear genes encoding photosynthesis-related proteins is dependent on plastid signals (Petracek et al., 1997; Sherameti et al., 2002; Tang et al., 2003). Plastid (redox) signals might also regulate the posttranslational import of proteins into chloroplasts (Jarvis and Robinson, 2004). Arabidopsis mutants defective in particular components of the import machinery show lower levels of proteins of the photosynthetic apparatus but unchanged or even enriched levels of other plastid-imported proteins (Bauer et al., 2000; Kubis et al., 2003), supporting the existence of substrate-specific

protein import pathways (Jarvis and Robinson, 2004). Finally, protein degradation within chloroplasts is also a substrate-specific process that can be modulated by the metabolic status of the plastid (Adam and Clarke, 2002).

Although more experiments are needed to identify the specific physiological signals that trigger the observed changes in MEP pathway enzyme levels and to clarify the role of plastome-encoded proteins in this process, our work represents an important step forward by revealing an influence of plastid-derived cues on the regulation of the MEP pathway in different plant species. The good correlation between DXR protein levels and FSM resistance in *rif10-1* and *rif10-1 35S:DXR* plants and the FSM resistance phenotype associated with increased DXS and DXR levels in mutant and CAP-treated Col seedlings suggest that these enzymes accumulate in an enzymatically active form and, therefore, that the observed changes in protein levels might be biologically relevant. Transgene-mediated regulation of the levels of DXS in Arabidopsis and tomato, DXR in peppermint (*Mentha piperita*), and HDR in Arabidopsis all resulted in concomitant changes in the levels of plastidial isoprenoid end products (Estévez et al., 2001; Mahmoud and Croteau, 2001; Botella-Pavía et al., 2004; Enfissi et al., 2005), suggesting that these three enzymes share some degree of control over the flux through the MEP pathway. The relative contribution of the rest of the MEP pathway enzymes remains to be established, but HDS has been proposed as another candidate to regulate flux (Querol et al., 2002; Rodríguez-Concepción et al., 2003). In the case of the *rif10* mutant, the up-regulation of flux-controlling enzymes of the MEP pathway does not result in higher levels of isoprenoid end products such as chlorophylls and carotenoids because the mutation also affects the formation of chloroplast structures that accommodate these photosynthetic pigments, as described above. But in wild-type chloroplasts, the synthesis of plastome-encoded proteins can be rapidly regulated by redox signals (with a major role of plastoquinone, a plastidial isoprenoid) in response to sudden changes in environmental conditions and photosynthetic activity (Pfannschmidt, 2002). It is therefore possible that plastome-mediated cues might modulate the accumulation of DXS, DXR, HDS, and HDR as a fine control of the MEP pathway which, unlike the coarse control exerted by changes in the expression of the nuclear genes, might allow individual chloroplasts to rapidly optimize the supply of isoprenoid precursors and meet their particular metabolic requirements.

MATERIALS AND METHODS

Plant Material

The activation-tagging T-DNA collections were purchased from the Nottingham Arabidopsis Stock Centre (NASC). Seeds from the Salk T-DNA insertion lines (Alonso et al., 2003) were also obtained from the NASC. The position of the inserted T-DNA in the genome of these mutants was confirmed by PCR. The *35S:DXS* and *35S:DXR* constructs were generated after cloning the full-length cDNAs encoding Arabidopsis (*Arabidopsis thaliana*) DXS and DXR into plasmid pBI221 (Clontech). Plasmid pCAMBIA1302 was used to generate *35S:GFP* lines. The plasmids were used for Agrobacterium-mediated transformation of Arabidopsis plants as described (Carretero-Paulet et al., 2002). For the identification of F_2 homozygous siblings segregating from the cross between *rif10-1* and *35S:DXR* plants, F_3 seeds were plated on medium supplemented with Basta (the resistance marker associated with the T-DNA used to generate the activation-tagging lines) or kanamycin (the resistance marker associated with the *35S:DXR* construct). Lines in which these markers did not segregate were identified as homozygous Col (sensitive to both Basta and kanamycin), *rif10-1* (only resistant to Basta), and *rif10-1 35S:DXR* (resistant to both Basta and kanamycin). All Arabidopsis genotypes used in this work were in the Col background. Seeds from tomato (*Lycopersicon esculentum*) and rice (*Oryza sativa*) were of the Microtom and Senia varieties, respectively.

Growth Conditions

Seeds were surface sterilized and germinated on petri dishes (Arabidopsis) or Magenta boxes (tomato and rice) with solid MS medium (Rodríguez-Concepción et al., 2004). After stratification for at least 2 d at 4°C, they 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 FSM (Gateway Chemical Technology), CAP (Sigma), or NFZ (Zorial). If required, plants were transferred from the plates to 1:1:1 (v/v) perlite:vermiculite: sphagnum soil mixture irrigated with mineral nutrients and grown in the LD chamber until seeds were produced.

Analysis of Mutant Phenotypes

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 FSM. The SE rate in the presence of the inhibitor was calculated relative to the value observed on plates without FSM (which was considered as 100%). Chlorophyll and carotenoid pigments were extracted, separated, and quantified as described (Rodríguez-Concepción et al., 2004).

Molecular Characterization of the Mutants

RNA and protein-blot analyses were carried out as described (Rodríguez-Concepción et al., 2001, 2004; Botella-Pavía et al., 2004; Guevara-García et al., 2005). A Bioanalyzer 2100 (Agilent Technologies) was used to monitor quantity and quality of RNA samples. Intensity of Coomassie-stained protein bands was quantified using a Molecular Imager densitometer (Bio-Rad). Since protein extracts from *rif10* and CAP-treated Col seedlings showed a decrease in the levels of RBCL (the major protein in these extracts), protein loading was normalized according to the levels of other proteins detected by Coomassie staining of the gels. The unspecific bands recognized by the anti-DXR serum were also used as an additional control of equal loading.

For the identification of the gene responsible for the FSM resistance phenotype in *rif10-1*, homozygous mutant plants were backcrossed with the Col wild type to test whether the corresponding mutation was linked to the presence of the only T-DNA detected in the mutant (as estimated from the associated Basta resistance marker). After identifying the recessive nature of the *rif10-1* mutation and its linkage to the T-DNA, the insertion site was identified using an inverse-PCR strategy. Genomic DNA was isolated from mutant seedlings as described (Carretero-Paulet et al., 2002), digested with *Bam*HI, and religated to create circular DNA molecules that were used as templates for PCR amplification with *Taq* DNA polymerase (Promega) and the T-DNA primers T7 (5′-TAATACGACTCACTATAGGG-3′), which anneals on the T7 promoter region next to the multicloning site of the pBluescript sequence, and BAR3R (5′-TGGGTTTCTGGCAGCTGG-3′), which anneals on the 3′-end region of the *BAR* gene conferring Basta resistance. Direct sequencing of the PCR products was carried out using the Big Dye Terminator cycle sequencing v2.0 kit of the ABI-PRISM system (Applied Biosystems) and primer ATC12 (5′-TTGGGCGGGTCCAGGG-5′), which anneals next to the left border of the T-DNA.

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LITERATURE CITED

Adam Z, Clarke AK (2002) Cutting edge of chloroplast proteolysis. Trends Plant Sci **7:** 451–456

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science **301:** 653–657

Baena-González E, Allahverdiyeva Y, Svab Z, Maliga P, Josse EM, Kuntz M, Maenpaa P, Aro EM (2003) Deletion of the tobacco plastid *psbA* gene triggers an upregulation of the thylakoid-associated NAD(P)H dehydrogenase complex and the plastid terminal oxidase (PTOX). Plant J **35:** 704–716

Bauer J, Chen K, Hiltbunner A, Wehrli E, Eugster M, Schnell D, Kessler F (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature **403:** 203–207

Botella-Pavía P, Besumbes O, Phillips MA, Carretero-Paulet L, Boronat A, Rodríguez-Concepción M (2004) Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. Plant J **40:** 188–199

Budziszewski GJ, Lewis SP, Glover LW, Reineke J, Jones G, Ziemnik LS, Lonowski J, Nyfeler B, Aux G, Zhou Q, et al (2001) Arabidopsis genes essential for seedling viability: isolation of insertional mutants and molecular cloning. Genetics **159:** 1765–1778

Carretero-Paulet L, Ahumada I, Cunillera N, Rodríguez-Concepción M, Ferrer A, Boronat A, Campos N (2002) Expression and molecular analysis of the Arabidopsis *DXR* gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the first committed enzyme of the 2-*C*-methyl-D-erythritol 4-phosphate pathway. Plant Physiol **129:** 1581–1591

Chappell J (1995) Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants. Annu Rev Plant Physiol Plant Mol Biol **46:** 521–547

Croteau R, Kutchan T, Lewis N (2000) Natural products (secondary metabolites). *In* R Jones, ed, Biochemistry and Molecular Biology of Plants. American Society of Plant Biologists, Rockville, MD, pp 1250–1268

Demmig-Adams B, Gilmore AM, Adams WW (1996) *In vivo* functions of carotenoids in higher plants. FASEB J **10:** 403–412

Eisenreich W, Bacher A, Arigoni D, Rohdich F (2004) Biosynthesis of isoprenoids via the non-mevalonate pathway. Cell Mol Life Sci **61:** 1401–1426

Eisenreich W, Rohdich F, Bacher A (2001) Deoxyxylulose phosphate pathway to terpenoids. Trends Plant Sci **6:** 78–84

Enfissi EMA, Fraser PD, Lois LM, Boronat A, Schuch W, Bramley PM (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. Plant Biotechnol J **3:** 17–27

Estévez JM, Cantero A, Reindl A, Reichler S, León P (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J Biol Chem **276:** 22901–22909

Estévez JM, Cantero A, Romero C, Kawaide H, Jiménez LF, Kuzuyama T, Seto H, Kamiya Y, León P (2000) Analysis of the expression of *CLA1*, a gene that encodes the 1-deoxyxylulose 5-phosphate synthase of the 2-*C*-methyl-D-erythritol-4-phosphate pathway in Arabidopsis. Plant Physiol **124:** 95–103

Green BR, Durnford DG (1996) The chlorophyll-carotenoid proteins of oxygenic photosynthesis. Annu Rev Plant Physiol Plant Mol Biol **47:** 685–714

Guevara-García A, San Román C, Arroyo A, Cortés ME, Gutiérrez-Nava ML, León P (2005) Characterization of the Arabidopsis *clb6* mutant illustrates the importance of posttranscriptional regulation of the methyl-D-erythritol 4-phosphate pathway. Plant Cell **17:** 628–643

Gutiérrez-Nava ML, Gillmor CS, Jiménez LF, Guevara-García A, León P (2004) *CHLOROPLAST BIOGENESIS* genes act cell and noncell autonomously in early chloroplast development. Plant Physiol **135:** 471–482

Jarvis P, Robinson C (2004) Mechanisms of protein import and routing in chloroplasts. Curr Biol **14:** R1064–R1077

Kasahara H, Hanada A, Kuzuyama T, Takagi M, Kamiya Y, Yamaguchi S (2002) Contribution of the mevalonate and methylerythritol phosphate pathways to the biosynthesis of gibberellins in Arabidopsis. J Biol Chem **277:** 45188–45194

Kubis S, Baldwin A, Patel R, Razzaq A, Dupree P, Lilley K, Kurth J, Leister D, Jarvis P (2003) The Arabidopsis *ppi1* mutant is specifically defective in the expression, chloroplast import, and accumulation of photosynthetic proteins. Plant Cell **15:** 1859–1871

Laule O, Furholz A, Chang HS, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange M (2003) Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana.* Proc Natl Acad Sci USA **100:** 6866–6871

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol **50:** 47–65

Mahmoud SS, Croteau RB (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. Proc Natl Acad Sci USA **98:** 8915–8920

Mandel MA, Feldmann KA, Herrera-Estrella L, Rocha-Sosa M, León P (1996) *CLA1*, a novel gene required for chloroplast development, is highly conserved in evolution. Plant J **9:** 649–658

Monde RA, Schuster G, Stern DB (2000) Processing and degradation of chloroplast mRNA. Biochimie **82:** 573–582

Nagata N, Suzuki M, Yoshida S, Muranaka T (2002) Mevalonic acid partially restores chloroplast and etioplast development in Arabidopsis lacking the non-mevalonate pathway. Planta **216:** 345–350

Pesaresi P, Varotto C, Meurer J, Jahns P, Salamini F, Leister D (2001) Knock-out of the plastid ribosomal protein L11 in Arabidopsis: effects on mRNA translation and photosynthesis. Plant J **7:** 179–189

Petracek ME, Dickey LF, Huber SC, Thompson WF (1997) Light-regulated changes in abundance and polyribosome association of ferredoxin mRNA are dependent on photosynthesis. Plant Cell **9:** 2291–2300

Pfannschmidt T (2002) Chloroplast redox signals: how photosynthesis controls its own genes. Trends Plant Sci **8:** 33–41

Querol J, Campos N, Imperial S, Boronat A, Rodríguez-Concepción M (2002) Functional analysis of the *Arabidopsis thaliana* GCPE protein involved in plastid isoprenoid biosynthesis. FEBS Lett **514:** 343–346

Rodríguez-Concepción M (2006) Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. Phytochem Rev (in press)

Rodríguez-Concepción M, Ahumada I, Diez-Juez E, Sauret-Güeto S, Lois LM, Gallego F, Carretero-Paulet L, Campos N, Boronat A (2001) 1-Deoxy-D-xylulose 5-phosphate reductoisomerase and plastid isoprenoid biosynthesis during tomato fruit ripening. Plant J **27:** 213–222

Rodríguez-Concepción M, Boronat A (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids: a metabolic milestone achieved through genomics. Plant Physiol **130:** 1079–1089

Rodríguez-Concepción M, Forés O, Martínez-García JF, González V, Phillips MA, Ferrer A, Boronat A (2004) Distinct light-mediated pathways regulate the biosynthesis and exchange of isoprenoid precursors during Arabidopsis seedling development. Plant Cell **16:** 144–156

Rodríguez-Concepción M, Querol J, Lois LM, Imperial S, Boronat A (2003) Bioinformatic and molecular analysis of hydroxymethylbutenyl diphosphate synthase (GCPE) gene expression during carotenoid accumulation in ripening tomato fruit. Planta **217:** 476–482

Sherameti I, Nakamura M, Yamamoto YY, Pfannschmidt T, Obokata J, Oelmüller R (2002) Polyribosome loading of spinach mRNAs for photosystem I subunits is controlled by photosynthetic electron transport. Plant J **32:** 631–639

Steinbacher S, Kaiser J, Eisenreich W, Huber R, Bacher A, Rohdich F (2003) Structural basis of fosmidomycin action revealed by the complex with 2-*C*-methyl-D-erythritol 4-phosphate synthase (IspC): implications

for the catalytic mechanism and anti-malaria drug development. J Biol Chem **278:** 18401–18407

Sugita M, Sugiura M (1996) Regulation of gene expression in chloroplasts of higher plants. Plant Mol Biol **32:** 315–326

Suzuki M, Kamide Y, Nagata N, Seki H, Ohyama K, Kato H, Masuda K, Sato S, Kato T, Tabata S, et al (2004) Loss of function of *3-hydroxy-3-methylglutaryl coenzyme A reductase 1* (*HMG1*) in *Arabidopsis* leads to dwarfing, early senescence and male sterility, and reduced sterol levels. Plant J **37:** 750–761

Tang L, Bhat S, Petracek ME (2003) Light control of nuclear gene mRNA abundance and translation in tobacco. Plant Physiol **133:** 1979–1990

Walter M, Kilian J, Kudla J (2002) PNPase activity determines the efficiency of mRNA 3′-end processing, the degradation of tRNA and the extent of polyadenylation in chloroplasts. EMBO J **21:** 6905–6914

Weigel D, Ahn JH, Blazquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrandiz C, Kardailsky I, Malancharuvil EJ, Neff MM, et al (2000) Activation tagging in Arabidopsis. Plant Physiol **122:** 1003–1013