Characterization of the Arabidopsis $clb6$ Mutant Illustrates the Importance of Posttranscriptional Regulation of the Methyl-\(\text{-}\)d-\(\text{-}\)Erythritol 4-Phosphate Pathway

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The biosynthesis of isopentenyl diphosphate and dimethylallyl diphosphate, the two building blocks for isoprenoid biosynthesis, occurs by two independent pathways in plants. The mevalonic pathway operates in the cytoplasm, and the methyl-\(\text{-}\)d-\(\text{-}\)erythritol 4-phosphate (MEP) pathway operates in plastids. Plastidic isoprenoids play essential roles in plant growth and development. Plants must regulate the biosynthesis of isoprenoids to fulfill metabolic requirements in specific tissues and developmental conditions. The regulatory events that modulate the plant MEP pathway are not well understood. In this article, we demonstrate that the CHLOROPLAST BIOGENESIS6 (CLB6) gene, previously shown to be required for chloroplast development, encodes 1-hydroxy-2-methyl-buteryl 4-diphosphate reductase, the last-acting enzyme of the MEP pathway. Comparative analysis of the expression levels of all MEP pathway gene transcripts and proteins in the $clb6$-1 mutant background revealed that posttranscriptional control modulates the levels of different proteins in this central pathway. Posttranscriptional regulation was also found during seedling development and during fosmidomycin inhibition of the pathway. Our results show that the first enzyme of the pathway, 1-deoxy-d-xylulose 5-phosphate synthase, is feedback regulated in response to the interruption of the flow of metabolites through the MEP pathway.

INTRODUCTION

Like all living organisms, plants synthesize an enormous variety of isoprenoids that serve as growth regulators, pigments, and structural components of membranes. Additionally, many isoprenoids are of biotechnological importance (Chappell, 2002). All isoprenoids are derived from two basic five-carbon precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Depending on the specific isoprenoid, these five carbon units undergo consecutive condensations and ulcer chemical modifications to produce the enormous variety of isoprenic compounds (Sacchetti and Poulter, 1997; Croteau et al., 2000).

In higher plants, two pathways are used for the synthesis of the basic isoprenoid units. The mevalonic (MVA) pathway occurs in the cytoplasm where sesquiterpenes (C$_{15}$) and triterpenes (C$_{30}$), such as phytosterols, dolichols, and farnesyl residues, for protein prenylation are produced (Bach et al., 1999; Lichtenthaler, 1999; Eisenreich et al., 2001). By contrast, the methyl-\(\text{-}\)d-\(\text{-}\)erythritol 4-phosphate (MEP) pathway operates in plastids and produces IPP and DMAPP (Figure 1) for the synthesis of isoprenoids, such as isoprene, carotenoids, plastoquinones, phyto conjugates (such as chlorophylls and tocopherols), and hormones (gibberellins and abscisic acid) (Schwender et al., 1996; Zeidler et al., 1997; Eisenreich et al., 1998; Lichtenthaler, 1999; Rohmer, 1999). In spite of this compartmentalization, evidence indicates that cross talk between both pathways exists (Kasahara et al., 2002; Bick and Lange, 2003; Hemmerlin et al., 2003; Laule et al., 2003), although the biological implications of this communication are not fully understood.

Through the valuable contributions of many laboratories, the elucidation of the entire MEP pathway has been accomplished in an impressively short time (Rohmer et al., 1996; Lichtenthaler, 1999; Eisenreich et al., 2001, 2004; Rodríguez-Concepción and Boronat, 2002). In the initial step of the pathway (Figure 1), the precursors pyruvate and glyceraldehyde 3-phosphate are converted to 1-deoxy-d-xylulose-5-phosphate (DXP) by the enzyme 1-deoxy-d-xylulose 5-phosphate synthase (DXS) (Rohmer et al., 1996; Sprenger et al., 1997; Lois et al., 1998). The DXP produced by this reaction is not used exclusively in the MEP pathway because it is also required for the production of the vitamins thiamin and pyridoxal (Julliard and Douce, 1991; Julliard, 1992). In the second step, DXP is converted to MEP by the enzyme DXP reductoisomerase (DXR). This represents the first committed step of the pathway, from which its name is derived (Kuzuyama et al., 1998; Takahashi et al., 1998; Schwender et al., 1999; Miller et al., 2000). The following steps involve five consecutive reactions (Figure 1) that culminate with the production of IPP
and DMAPP through the action of the enzyme 1-hydroxy-2-methyl-butanyl 4-diphosphate reductase (HDR) encoded by the *ISPH* gene (also *LytB*) (Lichtenthaler, 1999; Eisenreich et al., 2001; Rodríguez-Concepción and Boronat, 2002). The genes encoding each enzyme of the pathway have been identified in *Escherichia coli*, and in most cases the activity of the corresponding enzyme has been demonstrated (Hoeffler et al., 2002; Sauret-Gueto et al., 2003; Eisenreich et al., 2004).

The enzymes of the MEP pathway are highly conserved in evolution. The MEP pathway genes in *Arabidopsis thaliana* have high sequence similarity to the bacterial MEP pathway genes (Rodríguez-Concepción and Boronat, 2002). In the Arabidopsis genome, three genes that display sequence similarity to DXS are found. However, mutant analysis demonstrated that the disruption of one of those genes (CLA1) is sufficient to impair chlorophyll and carotenoid accumulation, suggesting a fundamental role of this gene for the functionality of the MEP pathway (Mandel et al., 1996; Araki et al., 2000; Estevez et al., 2000). Putative homologs of the *E. coli* genes that encode the downstream MEP enzymes are present as single copies in *Arabidopsis*. However, the functionality of all of these plant homologs has not been proven (Rohmer et al., 1996; Estevez et al., 2000; Carretero-Paulet et al., 2002; Hoeffler et al., 2002; Querol et al., 2002).

Currently, the regulatory aspects that govern the expression and activity of the enzymes in this pathway are almost unknown in plants. The isolation and characterization of mutants are useful tools not only to confirm the functionality of a particular gene, but also to analyze regulatory aspects of a metabolic pathway. For example, the use of mutants has been helpful in understanding some of the complex regulatory network that controls tetrapyrrole biosynthesis (Papenbrock and Grimm, 2001). MEP pathway mutants have also been used to uncover an active exchange of precursors between the cytosol and the plastids and helped to elucidate the contribution of the MEP pathway to the biosynthesis of specific isoprenoid compounds (Estevez et al., 2000; Kasahara et al., 2002; Gutierrez-Nava et al., 2004).

Recently, we have isolated and characterized a collection of mutant lines named *clb* (for chloroplast biogenesis) that affect early chloroplast development (Gutierrez-Nava et al., 2004). Here, we demonstrate that one of those albino mutants, *clb6-1*, carries a mutation in the Arabidopsis *ISPH* gene, encoding for the HDR enzyme (Figure 1). HDR is essential in *E. coli* and catalyzes the last step of the MEP pathway (Altincicek et al., 2001). This enzyme is able to produce a mixture of IPP and DMAPP both in vitro and in vivo (Adam et al., 2002; Altincicek et al., 2002; Hoeffler et al., 2002; Rohdich et al., 2002). A comparative expression analysis of all genes in this biosynthetic pathway at the mRNA and protein levels was performed in different mutant backgrounds and at different developmental stages. Coordinated regulation at the transcript level of all the genes in the pathway is observed in the mutants and during development. Interestingly, the levels of some of the proteins in the pathway do not reflect the levels and the regulation of their corresponding transcripts, indicating that posttranscriptional regulation plays an important role in controlling the MEP pathway. We show that one of the signals that triggers this novel posttranscriptional response is the level of the end products of this pathway, resulting in positive feedback regulation of DXS.

**RESULTS**

The **CLB6 Gene Corresponds to ISPH**

The loss of function of the MEP pathway genes have been shown to result in an albino phenotype that behaves non-cell autonomously (Mandel et al., 1996; Araki et al., 2000; Budziszewski et al., 2001; Crowell et al., 2003; Gutierrez-Nava et al., 2004). Based on the *clb6-1* albino phenotype (Figure 2B), *CLB6* was a good candidate to participate in the MEP pathway. In bacteria, *IsphL/LytB* encodes HDR, the enzyme that catalyzes the last step of the MEP pathway (Altincicek et al., 2001; Rohdich et al., 2002). Mapping experiments placed *CLB6* near the Arabidopsis homolog of the *ISPH* gene (Gutierrez-Nava et al., 2004). To corroborate this possibility, the *ISPH* gene from the *clb6-1* mutant line was
Expression Analysis of the MEP Pathway Genes in the clb6-1 Plant

To gain new insight into the regulatory mechanisms controlling the MEP pathway, we analyzed the impact of the clb6-1 mutation on the expression of all MEP pathway genes. Compared with wild-type seedlings, clb6-1 has lower transcript levels of all of the genes from the pathway (Figure 4A). The reduced levels of the transcripts appear unequal for all genes; DXR and ISPD are the most affected transcripts. Because this result was reproducibly observed in two independent experiments, we conclude that the transcript levels of the MEP genes appear to be sensitive to the blockage of the pathway or to the arrest of chloroplast development in this mutant.

Accumulation of the MEP Pathway Proteins in the clb6 Mutant

With the exception of the first two steps of the pathway that involve the DXS and DXR proteins (Lois et al., 2000; Estévez et al., 2001; Carretero-Paulet et al., 2002), basically nothing is known about the expression and regulation of the MEP pathway proteins in plants. Based on the impact that the clb6-1 mutant has on the expression levels of the MEP genes, it was of interest to analyze the steady state levels of their corresponding proteins. To perform this analysis, polyclonal antibodies raised against glutathione S-transferase (GST) fusion proteins of the 4-diphosphocytidyl-2C-methyl-d-erythritol 4-phosphate synthase (CMS), the 4-diphosphocytidyl-2C-methyl-d-erythritol kinase (CMK), and the 2C-methyl-d-erythritol 2,4-diphosphate synthase (MCS) proteins (Figure 1) were generated. Specific antibodies for the rest of the enzymes of the pathway include one against DXS (Estévez et al., 2000) and three others against the DXR, 2C-methyl-d-erythritol 2,4-cyclodiphosphate reductase (HDS), and HDR proteins (Figure 1). Using this complete set of antibodies, the level of the MEP proteins was compared in 10 μg of total protein extracts from 18-d-old mutant and wild-type seedlings. Experimental immunodetection conditions for each of the antibodies were optimized to be under a linear detection range by analyzing serial dilutions of total protein in 18-d-old wild-type extracts (Figure 4C). In agreement with the transcript analysis (Figure 4A), no HDR protein was detected in clb6-1, which supports the conclusion that it is a null mutant allele (Figure 4B). In addition, this data also corroborated the specificity of the HDR antibody.

The low transcript abundance observed for the MEP genes (Figure 4A) is reflected in low to almost undetectable protein levels for the DXR, CMK, and MCS proteins in the clb6-1 mutant (Figure 4B). However, in spite of the low transcript levels of the ISPD and ISPG genes (Figure 4A), their corresponding protein level (CMS and HDS) was similar compared with wild-type plants (Figure 4B). Interestingly, the most striking difference reproducibly observed between the transcript and protein abundance corresponds to the DXS/CLA1 gene. Whereas the DXS transcript is notably reduced in the clb6-1 mutant (Figure 4A), the level of the DXS protein is substantially increased (more than 10-fold) compared with the wild-type level (Figure 4B). Because of the high level of expression of the DXS protein in the clb6-1 mutant, it...
Figure 3. Molecular Characterization of the ISPH Gene in the clb6-1 Mutant and in Complemented Plants.

(A) Schematic representation of the structure of the ISPH gene from Arabidopsis. The wild-type ISPH gene sequence showed the presence of a
was important to demonstrate that the detection conditions used in this experiment were still under a linear range. This was done by analyzing serial dilutions of the same clb6-1 mutant protein extracts with each of the antibodies raised against the MEP proteins (with the exception to those against MCS and HDR proteins because they are not detected in this mutant) using the same immunodetection conditions (Figure 4C). The specificity of each antibody was corroborated as described in Methods. The polyclonal antibody used against HDS reproducibly detects two bands with molecular weight similar to the one expected for HDS. The identity of the HDS as the lower band (marked by an arrow) was previously demonstrated by analyzing two mutant alleles for this enzyme in which this band is absent (Gutiérrez-Nava et al., 2004). The upper band is an unspecified signal present in the preimmune serum (data not shown).

To analyze whether the accumulation of the DXS protein is a particular response of the clb6-1 mutant, the level of the DXS protein in other mutants that affect the MEP pathway was also determined. The analysis included the clb1-1 mutant (Mandel et al., 1996; Estévez et al., 2000), a mutant in the DXR gene (line 4036) (Budziszewski et al., 2001), a mutant in the ISP gene (line SALK_030640), and a recently described mutant (clb4-1) that affects the ISP gene (Gutiérrez-Nava et al., 2004). As shown in Figure 5A, the protein accumulation pattern in these independent MEP mutants was similar to that observed for clb6-1. The abundance of the CMS protein was similar to that in the wild-type plant. However, increased protein levels were detected for DXS and HDR compared with the wild type (Figure 5A). These increments were also corroborated to be under linear detection conditions (see Supplemental Figure 1A online). Once again the most remarkable difference in protein accumulation found between the wild type and the different MEP mutants corresponds to DXS. Taken together, these results indicate that some of the MEP pathway proteins are posttranscriptionally regulated by their translation or degradation.

It is well established that the developmental and metabolic status of the chloroplast impacts the expression of a wide variety of nuclear genes by an active communication between plastids and the nucleus (Papenbrock and Grimm, 2001; Surpin et al., 2002). Such a regulatory process permits coordination between nuclear gene expression and the requirements of organelle function and development. However, the nature of the signals and mechanism of this retrograde regulation is far from understood. All of the mutants in the MEP pathway display an albino phenotype, and previous reports have demonstrated that the chloroplasts in several of these mutants are arrested at an early developmental stage. In addition, the expression of some nuclear-encoded photosynthetic genes is downregulated in this type of mutant (Mandel et al., 1996; Araki et al., 2000; Budziszewski et al., 2001; Gutiérrez-Nava et al., 2004). Thus, it was important to discriminate whether the accumulation of the DXS protein observed in the MEP mutants could be a general consequence of an arrest in chloroplast development. To accomplish this, we took advantage of the clb5-1 mutant, in which chloroplasts are arrested early in development, similar to the MEP mutants (Gutiérrez-Nava et al., 2004). Although the identity of the CLB5 gene is still unknown, mapping data showed that this mutant does not correspond to any of the structural genes of the MEP pathway (Gutiérrez-Nava et al., 2004). The accumulation of the DXS and HDR proteins found in the different MEP mutants is no longer observed in clb5-1, in which both proteins are present at lower levels than in wild-type plants (Figure 5A). This result strongly suggests that the accumulation of DXS and HDR is not a general consequence of an arrest in chloroplast development, but probably a more specific response as a result of the blockage of the MEP pathway.

To further support this idea, the accumulation of MEP proteins was analyzed in an additional photosynthetic mutant (ch1-2). ch1-2 results in reduced levels of chlorophyll b by affecting the chlorophyll a oxygenase, which is required for chlorophyll b
synthesis (Espineda et al., 1999). It has been shown that the expression of some nuclear photosynthetic proteins is affected in this mutant (including the LHCB1 and a redox-controlled thylakoid kinase), probably as a result of a retrograde regulation. As shown in Figure 5B, the level of the MEP proteins in the 18-d-old ch1-2 mutant is similar to that of their corresponding wild type at the same age. The transcript levels of DXS, DXR, and ISPH genes were also similar in this mutant compared with wild-type

Figure 4. Expression Analysis of the MEP Pathway Genes in the clb6-1 Mutant.

(A) RNA gel blot analyses of DXS, DXR, ISPD, ISPE, ISPF, ISPG, and ISPH transcript accumulation in 18-d-old wild-type Ler (Wt) and clb6-1 plants grown on MS medium. Five micrograms of total RNA were loaded in each lane and hybridized with the entire ISPH cDNA. The methylene blue–stained membrane (MB) is shown as a loading control. These RNA gel blots are representative of two independent biological experiments.

(B) Expression analysis of the MEP enzymes in wild-type (Wt) and clb6-1 mutant. Total protein extracts were isolated from 18-d-old Ler and clb6-1 plants. Immunoblots were performed with antibodies against the specified enzymes (Figure 1). Each lane contains 10 µg of total protein extract. Immunodetection was done using a 1:1000 dilution of the DXS antibody, 1:1500 of DXR, CMK, MCS, HDS, and HDR antibodies, and 1:2500 of the CMS antibody. A Coomassie blue–stained gel (Coo.) run in parallel is shown as a loading control. The arrow marks the specific HDS protein. The protein gel blots shown are representative from three independent biological replicates.

(C) and (D) Linearity of the antibody reactions was corroborated by protein gel blots testing serial dilutions of the same 18-d-old Ler wild-type (C) or clb6-1 extracts (D) used in (B). Each lane contains 20, 10, 5, and 2.5 µg as indicated. Immunoblots were performed with antibodies raised against DXS, DXR, CMS, CMK, and HDS using the same dilution as in (B) and as specified in Methods. The clb6-1 mutant control does not include MCS and HDR proteins because they are not detected in these extracts.

Figure 5. Expression Analysis of the MEP Pathway Proteins in Different Photosynthetic Mutants.

(A) Total protein extracts were isolated from 18-d-old Salk_030640 line affected in ISPD (ispD), a mutant for ISPG (clb4-1), a mutant of DXR (dxr) (line 4036, accession number AB009053), the wild type (Wt), clb1-1 (clb1), clb6-1, and clb5-1 seedlings. For simplification, the wild type presented in the figure corresponds to the Ler ecotype, but similar protein levels were detected in the Wassilewskija (background for clb1-1) and Col-0 (background for ispD and dxr) ecotypes. Immunoblots were performed as described in the legend of Figure 4B. A Coomassie blue–stained gel (Coo.) run in parallel with the same samples is shown as a loading control. The asterisks mark the DXS and HDR proteins that accumulate in the MEP mutants. The level of the MEP proteins shown in these protein gel blots is representative of two independent biological experiments.

(B) Protein gel blot analysis of the MEP pathway proteins in ch1-2 chlorina mutant. Total protein extracts were isolated from 18-d-old Col-0 wild-type (Wt), ch1-2 (ch1), and clb6-1 (clb6) mutants. Immunoblots were performed against antiserum made against all MEP proteins. Each lane contains 10 µg of total protein, and the antibodies were used to the same dilution as described in the legend of Figure 4B. The Coomassie blue–stained gel (Coo.) is shown as a loading control. The asterisks mark the DXS and HDR proteins that accumulate in the MEP mutants. The level of the MEP proteins shown in these protein gel blots is representative of two independent biological experiments.

(C) and (D) Linearity of the antibody reactions was corroborated by protein gel blots testing serial dilutions of the same 18-d-old Ler wild-type (C) or clb6-1 extracts (D) used in (B). Each lane contains 20, 10, 5, and 2.5 µg as indicated. Immunoblots were performed with antibodies raised against DXS, DXR, CMS, CMK, and HDS using the same dilution as in (B) and as specified in Methods. The clb6-1 mutant control does not include MCS and HDR proteins because they are not detected in these extracts.

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plants (Figure 5C). This result also supports our conclusion that the increase in the protein level of some MEP enzymes, such as DXS, is a particular feature of mutants that affect the MEP pathway. This regulation could have a physiological significance that allows for an increase in the DXS protein level to counteract its low transcript abundance. This result is relevant because DXS has been suggested to be a limiting enzyme for the MEP pathway in plants (Lois et al., 2000; Estévez et al., 2001).

Developmental Expression of the MEP Pathway

One major difference between the wild-type and the albino mutant plants used in the previous experiment was their developmental stage. Whereas 18-d-old wild-type plants already have two or three fully expanded pairs of leaves, the albino mutants have only one pair of expanding leaves. To control for this difference, the accumulation of DXS protein was evaluated in two developmental stages. One includes seedlings with fully expanded cotyledons (Figure 6A), corresponding to 3 d old in wild-type seedlings and 7 d old in the albino mutants. The other corresponds to seedlings at the emergence of the first true leaves (Figure 6B) that corresponds to 8 d old in the wild type and 13 d old in the albino mutants. As shown in Figure 6B, the difference in the DXS in the DHS and HDR levels detected between 18-d-old wild-type and mutant seedlings is also detected in seedlings at the first true leaves stage in two different MEP mutants, but to a lesser extent. However, in three independent experiments, the level of DXS and HDR protein levels at the cotyledon stage in mutant plants were not higher than the wild-type seedlings (Figure 6A). This result indicates that the difference in the protein accumulation levels between the wild type and the MEP mutants is partially due to the different developmental stage of these plants. However, this is not the entire explanation because the accumulation of the DXS and HDR proteins in the clb5-1 albino mutant is still much lower than the wild-type seedlings in both developmental conditions. Thus, these results demonstrate that the levels of DXS, DXR, and HDR exhibit important fluctuations during plant development (3, 8, and 18 d).

To further analyze the expression levels of the MEP proteins during development and to explore whether or not posttranscriptional regulation could also account for the accumulation in the 3-d-old wild-type seedlings, we conducted a comparative analysis of all the MEP transcripts and proteins in different developmental stages. As shown in Figure 6C, the transcript abundance of all genes of the MEP pathway is low in young seedlings at the cotyledon stage (3 d old). This is followed by a substantial increase for all the MEP gene mRNAs between 3 and 6 d. This coordinated accumulation appears to be coincident with the emergence and expansion of the first true leaves in the seedling. Later in development (18 d old), the transcript level for most genes is higher compared with the 3-d-old seedlings, but unique responses for each gene are observed. For example, the maximum transcript level of the DXS, DXR, and ISPF genes is found in the 6-d-old seedlings, which is in agreement with previously reported data of DXS/CLA1 (Estévez et al., 2000). However, a different accumulation pattern is observed for the ISPG gene, as the level of its transcript is similar in the 6- and 18-d-old seedlings (Figure 6C). Finally, the transcript levels of the

![Figure 6. Expression Analysis of the MEP Pathway Genes during Seedling Development.](image-url)

**A** Protein accumulation in young wild-type and mutant seedlings. Seedlings of *Ler* wild-type (Wt) and mutant plants were harvested at the cotyledon stage that corresponds to 3 d for the wild type and 7 d for *clb4-1*, *clb6-1*, and *clb5-1* mutants.

**B** The first true leaves expansion stage corresponds to 8 d old for wild type (Wt) and 13 d old for *clb4-1*, *clb6-1*, and *clb5-1* plants. For comparison, a picture of the wild-type and clb5-1 seedlings for each developmental stage is shown. *clb4* and *clb5* were harvested at a same developmental stage. Total protein was extracted from each sample, and 10 μg were loaded in each lane. Immunoblots were performed using specific antibodies against the DXS, DXR, and HDR proteins. The conditions for the immunodetection were as described in the legend of Figure 4B. A Coomassie blue–stained gel (Coo.) with equal loading is shown as a control. The level of the MEP proteins shown in these protein gel blots is representative of two independent biological experiments.

**C** RNA gel blot analysis of the MEP genes during development. *Ler* wild-type seedlings were grown for 3, 6, and 18 d. Each lane contains 5 μg of total RNA. Blots were hybridized with the indicated probes. The methylene blue–stained (MB) membrane is shown as a loading control. The level of the MEP transcripts observed in these blots is representative of two independent experiments.

**D** Protein accumulation of the MEP enzymes during development. *Ler* wild-type seedlings were harvested at 3, 6, 10, 18, and 30 d after transfer to the growth chamber. Each lane contains 10 μg of the total protein. Immunoblots were performed using specific antibodies raised against the DXS, DXR, CMS, CMK, MCS, HDS, and HDR proteins using the antibody dilutions described in the legend of Figure 4B. Corroboration of linearity was done using extracts from the 6-d-old seedlings as the highest MEP protein levels were detected at this stage (see Supplemental Figure 1B online). A Coomassie blue–stained gel (Coo.) with equal loading is shown as a control. The arrow indicates the specific band for the HDS protein. The protein levels shown in the blots were reproducible in two independent experiments.
ISP, ISPE, and ISPH genes are even higher in 18-d-old plants (Figure 6C).

To compare the developmental regulation of the MEP transcripts with their corresponding protein levels, protein gel blots were performed with total protein extracts from plants at different developmental stages. Interestingly, in spite of significant changes in the mRNA transcript abundances between 3- and 6-d-old seedlings for all MEP genes (Figure 6C), the levels of their corresponding proteins remain mostly constant (Figure 6D). A discrete increase in the protein steady state abundance can be observed for DXR, CMK, and MCS during development (Figure 6D). However, this is not the case for the DXS, CMS, HDS, and HDR proteins, whose levels are similar or even higher at 3 d compared with the 6- and/or 10-d-old plants. Specifically for DXS, the highest protein level is found at the 3-d-old stage, which corresponds to the lowest level of its transcript. This demonstrates that posttranscriptional events appear to also regulate the level of the DXS protein during early development.

The accumulation of the MEP pathway proteins by this posttranscriptional regulation might constitute a regulatory mechanism that could play an important role to adjust for cellular requirements. One such possibility is the demand of the final MEP products, IPP and DMAPP. These metabolites must be very low in the different MEP mutants and might also be limiting during seedling development as a result of the demand for different isoprenoid products, such as chlorophyll and carotenoid pigments, that have an important accumulation in a 3-d developmental period (Table 1). To explore this possibility, the accumulation of all MEP proteins in the presence of a specific inhibitor of the pathway was determined. We used the antibiotic fosmidomycin, which inhibits the activity of the DXR protein and reduces the levels of the final products derived from the MEP pathway (Schwender et al., 1999; Laule et al., 2003). To uncouple this response from the chloroplast developmental arrest that exists in the MEP mutants, the fosmidomycin treatment was performed for a restricted period of time. Wild-type seedlings were grown for 8 d in MS media and then transferred to a media with or without 100 μM fosmidomycin. This treatment was also analyzed in the presence of the carotenoid biosynthesis inhibitor norflurazon (Oelmüller, 1989). Under high light growth conditions, this herbicide promotes a massive internal photoxidative damage of the chloroplast. This damage results in an albino phenotype and also severely affects the expression of a subset of nuclear-encoded photosynthetic genes (Oelmüller, 1989) but does not directly interfere with the MEP biosynthetic pathway. Using protein gel blot analysis, the level of the MEP proteins from seedlings grown without or in the presence of 5 μM norflurazon was compared. This analysis was performed in Ler wild-type seedlings grown up to the emergence of the first true leaves because at this developmental stage we have previously observed clear differences in the DXS accumulation between wild-type and MEP mutant plants (Figure 6B). As shown in Figure 8A, a decrease in the level of protein accumulation of all the MEP proteins is observed in the presence of norflurazon. This contrasts with the accumulation observed for the DXS in the albino mutants at a similar developmental stage (Figure 6B). Thus, this result supports the hypothesis that the accumulation of the DXS protein in the albino mutants at a similar developmental stage corresponds to the lowest level of its transcript. This demonstrates the efficiency of the fosmidomycin treatment. No other significant differences were observed for any other proteins in the pathway within the period analyzed (Figure 7A; see Supplemental Figure 2 online), which suggests that this regulation might be specific for the DXS protein.

As shown in Figure 7C, the increase in the DXS protein levels in response to the inhibitor (Fos –) can not be explained by changes in the transcript levels because no differences were observed when compared with the corresponding control (Fos +). It is worthy to note that lower DXS transcript levels were reproducibly detected in the control and fosmidomycin-treated plants at 6 h. This response appears to be intrinsic to the experimental procedure. The fosmidomycin treatment results in the accumulation of DXS protein, a very similar response to the one observed with the MEP mutants (Figure 5A). Thus, this result can be interpreted that the level of DXS protein is regulated by a translational and/or posttranslational control in response to the MEP pathway flux rate.

To further support this idea, the expression level of the MEP proteins was also analyzed in the presence of the carotenoid biosynthesis inhibitor norflurazon (Oelmüller, 1989). Under high light growth conditions, this herbicide promotes a massive internal photoxidative damage of the chloroplast. This damage results in an albino phenotype and also severely affects the expression of a subset of nuclear-encoded photosynthetic genes (Oelmüller, 1989) but does not directly interfere with the MEP biosynthetic pathway. Using protein gel blot analysis, the level of the MEP proteins from seedlings grown without or in the presence of 5 μM norflurazon was compared. This analysis was performed in Ler wild-type seedlings grown up to the emergence of the first true leaves because at this developmental stage we have previously observed clear differences in the DXS accumulation between wild-type and MEP mutant plants (Figure 6B). As shown in Figure 8A, a decrease in the level of protein accumulation of all the MEP proteins is observed in the presence of norflurazon. This contrasts with the accumulation observed for the DXS in the albino mutants at a similar developmental stage (Figure 6B). Thus, this result supports the hypothesis that the

### Table 1. Chlorophyll and Carotenoid Content of Wild-Type Seedlings during Development and Treated with Fosmydomicin

<table>
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<th>Samples</th>
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<th>Chlorophyll b</th>
<th>Total Chlorophyll</th>
<th>Carotenoids</th>
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<td>3-d-old Ler</td>
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<td>48.3 ± 22.5 (100)</td>
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<td>6-d-old Ler</td>
<td>368.9 ± 12.6 (288.3)</td>
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<td>503.5 ± 13.8 (286.4)</td>
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<td>18-d-old Ler</td>
<td>521 ± 35.2 (407)</td>
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<td>198.3 ± 20.9 (328.5)</td>
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<tr>
<td>8-d-old Ler MS Fos –</td>
<td>280 ± 0.71 (100)</td>
<td>79.2 ± 27.9 (100)</td>
<td>358.1 ± 27.1 (100)</td>
<td>99.8 ± 3.7 (100)</td>
</tr>
<tr>
<td>8-d-old Ler MS Fos +</td>
<td>127.4 ± 37 (45.5)</td>
<td>25.6 ± 7.2 (32.3)</td>
<td>152.5 ± 29.7 (42.6)</td>
<td>41.6 ± 6.8 (41.7)</td>
</tr>
</tbody>
</table>

aPigments were extracted from seedlings and quantified as described in Methods. Values are expressed in micrograms of pigment per gram of fresh weight. The mean of two replicates is shown ± SE. In parentheses is shown the percentage of pigments in relation to the 3-d-old seedling or the wild-type without fosmidomycin (taken as 100%). The pigment level shown in this table corresponds to the 48-h treatment in liquid MS media (Fos –) or in liquid MS media plus fosmidomycin (Fos +).
increase in the level of DXS protein in the MEP mutants and in the fosmidomycin treatment results from the blockage of the MEP pathway flow and is not an indirect effect of an arrest in chloroplast development. The lower level of the MEP proteins appears to reflect the low level of their corresponding transcripts, at least in the case of DXS, DXR, and HDR, which are dramatically reduced in the norflurazon treatment (Figures 8B and 8C).

DISCUSSION

This study addresses the characterization of a mutant of the MEP pathway and a comparative analysis of the complete MEP pathway at the transcript and protein levels in mutants and during seedling development.

clb6, a Mutant That Affects the Last Gene of the MEP Pathway

Arabidopsis in planta complementation and expression analysis demonstrated that the albino mutant clb6-1 contains a null mutant allele of the ISPH gene, required for the last biosynthetic step of the plastidic MEP isoprenoid pathway. This gene codes for the enzyme named HDR, responsible for the branch point in the conversion of hydroxymethylbutenyl 4-diphosphate into a 5:1 mixture of IPP and DMAPP (Adam et al., 2002; Altincicek et al., 2002; Hoeffler et al., 2002; Rohdich et al., 2002). This biosynthetic step is distinct from the cytosolic mevalonate pathway, in which the DMAPP is synthesized by an isomerase type of enzyme at a later step after IPP synthesis (Hamano et al., 2001; Rodríguez-Concepción and Boronat, 2002). The precise mechanism of this unique branching is still being studied, but recent evidence supports that such a branching is also present in higher plants (Hoeffler et al., 2002). The molecular characterization of the clb6-1 mutant revealed a single base pair insertion that results in a frame shift creating a stop codon. Although this mutation should result in a truncated protein, RNA and protein gel blot analyses demonstrated that neither the ISPH transcript nor the HDR protein is detectable in the clb6-1 mutant. The absence of the ISPH transcript in the clb6-1 mutant could result from a nonsense-mediated mRNA decay mechanism described for other genes in plants (Isshiki et al., 2001; Byres, 2002). Thus,

Figure 7. Accumulation of the MEP Proteins in Fosmidomycin-Treated Seedlings.

(A) Arabidopsis Ler wild-type seedlings were grown for 8 d on filter paper saturated with MS liquid medium. Treatment was done by the addition of MS media for controls (−) or MS media supplemented with 100 µM fosmidomycin (+). Samples were collected at 0, 6, 24, and 48 h after treatment. The level of the MEP proteins was analyzed by protein gel blots using specific antibodies against each protein as described in the legend of Figure 4B. Ten micrograms of total protein was loaded in each lane. A Coomassie blue–stained gel (Coo.) with equal loading is shown as a control. A representative experiment from three independent biological experiments is shown.

(B) Comparative graph of the densitometric quantification for the DXS protein level in response to fosmidomycin treatment. The DXS protein level from the samples treated without (− Fos, dashed line) or with (+ Fos, solid line) fosmidomycin was obtained by densitometric analysis at each time point. The densitometric quantification was normalized to the level of an arbitrary protein from the Coomassie blue–stained gel (arbitrary units) and referred to the DXS protein level at 0 h, taken as 1. The data on the graph correspond to the mean ± se of three independent experiments.

(C) Relative levels of expression of the DXS gene after fosmidomycin treatment. The samples used in the RNA gel blot analysis were the same as in the protein gel blots. Each lane contains 5 µg of total RNA. Hybridization was performed using a specific probe for the DXS gene. The methylene blue–stained membrane (MB) is shown as a loading control.
homolog but with the *E. coli* counterpart (Lange et al., 2000). Thus, it has been suggested that lateral gene transfer might have played a critical role in the acquisition of several of their MEP pathway genes (Boucher and Doolittle, 2000; Lange et al., 2000).

To analyze the particular case of HDR, a phylogenetic tree was constructed. In contrast with other MEP pathway proteins, the HDR enzyme from Arabidopsis and other plants branches closer to its cyanobacterial counterpart, supporting a common origin (Figure 9). A different type of tree is observed using the DXR enzyme (Lange et al., 2000; see Supplemental Figure 3 online). Previous analysis considered that DXR represented the only gene of the MEP pathway to have evolved directly from the plastid ancestor (Lange et al., 2000). Based on our phylogenetic analysis, we suggest that the DXR and ISPH genes might have a similar evolutionary origin.

### Regulation of the MEP Pathway Transcript Levels

The comparative analysis performed in this study demonstrates that the MEP pathway genes are subject to several levels of regulation that modulate both transcript and protein levels. It was found that the transcript level of all the genes of the pathway are diminished in the *clb6-1* mutant compared with the wild type. This result is in agreement with previous expression analysis of the MEP pathway genes in the *cla1-1* mutant, in which most of these genes were found to be drastically reduced (Laule et al., 2003). Previous work showed that chloroplasts in the *cla1-1* and *clb6-1* mutants arrest early in development, and the expression of several photosynthetic nuclear- and chloroplast-encoded genes is low in these mutant plants (Mandel et al., 1996; Sundberg et al., 1997; Gutiérrez-Nava et al., 2004). Different studies have demonstrated that signals generated from the arrested chloroplasts alter the expression of a specific subset of nuclear-encoded chloroplast proteins (Surpin et al., 2002; Larkin et al., 2003; Strand et al., 2003). Thus, we hypothesize that the general decrease of the different MEP gene transcript levels is likely a consequence of the plastid arrest in these mutants. In this work, this idea is supported by the observation that the transcript level of several MEP genes is low in the *clb5-1* albino mutant and in wild-type seedlings grown in the presence of the carotenoid biosynthesis inhibitor norflurazon, used as a tool to investigate nuclear and plastid interactions (Oelmüller, 1989; Strand et al., 2003).

Experimental evidence has demonstrated the existence of different signaling pathways involved in this retrograde regulation that are mediated by signals such as tetrapyrroles and redox (Surpin et al., 2002). The expression level of several MEP genes is not diminished in the *cht1-2* mutant (Figure 5C), which is affected in the expression of some nuclear genes, presumably in relation to a redox control (Espineda et al., 1999; Surpin et al., 2002). Thus, we favor that the signal that mediates the downregulation of the MEP transcript genes might originate early during chloroplast development, including the gun signal, but it is probably not mediated by the redox signal(s). Two independent studies based in microarray analysis have found that a substantial number of nuclear genes are downregulated as a consequence of a block in chloroplast development by norflurazon (Strand et al., 2003; A. McCormac and M. Terry, NASCArrays/Gene Swinger, http://affymetrix.arabidopsis.info/narrays/geneswinger.pl?experimentid=...
Remarkably, in both of these analyses the expression of the DXS/CLA1 gene was not affected by the norflurazon. Based on our RNA gel blot and RT-PCR data, this gene is clearly downregulated in the presence of the same concentration of norflurazon used in the microarray analyses (Figures 8B and 8C). By contrast, and in agreement with our data, in the most recent of these studies, other genes from the pathway, including DXR, ISPG, and ISPF, are reported to be downregulated in response to chloroplast photodamage. This observation correlates with our RT-PCR data (Figure 8C) and supports that at least some of the MEP pathway genes are targets of retrograde regulation.

This analysis also shows that the transcript levels of all MEP genes are modulated during development, displaying their lowest levels at early developmental stages (3 d). This developmental regulation has been previously reported for the DXS and DXR genes (Estévez et al., 2000; Carretero-Paulet et al., 2002). Coordinated accumulation of the transcript levels for all the genes of the pathway is observed in early development, and it appears to occur during a precise developmental window (between 3 and 6 d). This suggests that a common regulatory mechanism could be responsible for such a response. It was observed that this transcript accumulation is coincident with an important increase in the levels of chlorophyll and carotenoid pigments in the same developmental period (Table 1). Thus, we speculate that this regulation might be one of the key steps that controls proper responses for a higher synthesis of these products under particular developmental conditions. Later in development, the maximum transcript levels and their accumulation kinetics differ for each gene, suggesting the existence of additional specific regulatory mechanisms.

The MEP Pathway Is Regulated by Posttranscriptional Mechanisms

This study demonstrates that the accumulation of the MEP pathway proteins is also regulated by a posttranscriptional (translational and/or posttranslational) mechanism. The comparative analysis of levels of MEP transcripts for DXS, ISPD, ISPG, and ISPH and their corresponding proteins showed that mRNA and protein levels do not always correspond (Figures 4, 6, and 7). Such regulation is observed in different mutants of the pathway, and it does not appear to be a consequence of retrograde
regulatory events because it is not observed in an unrelated albino mutant (clb5-1) or in seedlings grown in the presence of norflurazon. Posttranscriptional regulatory events were also observed during seedling development. Among all the MEP proteins, the most evident posttranscriptional regulation is for DXS, and it was studied in more detail. For example, the steady state level of DXS in different MEP mutants is more than 10-fold higher than in wild-type plants, and its protein abundance at early stages (3 to 6 d) is higher than later in development (18 d). Several in vivo studies support that DXS has a limiting role for isoprenoid biosynthesis in different organisms, including bacteria and plants (Kuzuyama et al., 2000; Miller et al., 2000; Estévez et al., 2001; Carretero-Paulet et al., 2002). Thus, the posttranscriptional accumulation of this protein could represent a key regulatory event that might have an impact on the flow of the entire pathway.

Modulation of the transcript and protein levels or the enzymatic activity by the product(s) of a biosynthetic pathway is not an uncommon regulatory mechanism. It has been shown that hydroxymethylglutaryl CoA reductase, a key enzyme for the MVA pathway, is regulated by a feedback mechanism in animals and plants by end products of the pathway (Goldstein and Brown, 1990; Wentzinger et al., 2002; Hemmerlin et al., 2003). Unfortunately, the activity of the individual MEP pathway enzymes has not been successfully measured in plants yet. Thus, the biological impact of this regulation on the DXS enzymatic activity remains to be shown. However, the fact that DXS protein posttranscriptional regulation is observed only in mutants blocked in the MEP pathway allows us to hypothesize that this regulation could be triggered by a demand of the pathway products. This idea was further supported by the high DXS protein accumulation observed during early development that correlates with an important increase in chlorophyll and carotenoid content (Table 1). Finally, posttranscriptional accumulation of DXS was observed in response to a perturbation of the pathway flow with the use of the selective MEP pathway inhibitor fosmidomycin (Kuzuyama et al., 1998; Zeidler et al., 1998). The fosmidomycin treatment conditions used in our analysis were based in previous literature reports and were chosen to diminish secondary effects of this herbicide as a result of chloroplast phototoxicative damage (Rojdríguez-Concepción et al., 2001; Okada et al., 2002; Laule et al., 2003). Remarkably, this posttranscriptional DXS protein accumulation was not observed in the presence of norflurazon. This result is consistent with the idea that although this herbicide results in an albino phenotype and in chloroplast arrest, it should not directly impair IPP and DMAPP synthesis as it inhibits specifically carotenoid biosynthesis.

In addition to DXS, our mutant and developmental analyses indicate that the accumulation of other proteins of the MEP pathway appear also to be under translational and/or posttranslational control. However, the biological significance of these regulatory events will require future analysis. In contrast with DXS, the accumulation of CMS, HDS, and HDR proteins does not appear to be sensitive to the pathway flow. The levels of these proteins do not accumulate in a significant way during the fosmidomycin treatment, at least over the period and conditions analyzed. Previous work by Carretero-Paulet et al. (2002) reported a transient accumulation (within 12 h of treatment) of the DXR protein after fosmidomycin treatment, without a significant change in DXS level. In our experiments, we did not observe an increase in the level of the DXR protein, nor did we observe a decrease in the DXS levels even after 48 h of treatment (Figures 7A and 7B; see Supplemental Figure 2 online). Further experiments will be needed to evaluate the cause of this discrepancy.

The signal(s) and the mechanism(s) responsible for posttranscriptional regulation of DXS are presently unknown. The fact that the accumulation of the DXS protein is observed in mutants affected in different biosynthetic steps of the MEP pathway makes unlikely that intermediate molecules of this pathway could play a role in the signaling mechanism. Possible candidates are the final products of the pathway, IPP and/or DMAPP, that have also been suggested to be involved in the cross talk between the cytosolic MVA and the plastid MEP pathway (Bick and Lange, 2003; Hemmerlin et al., 2003). However, other downstream metabolites cannot be excluded. This regulation could be important in modulating the flow of products through the pathway, without risking potentially deleterious overproduction or underproduction of some end products.

In conclusion, our study has demonstrated that similar to other biosynthetic pathways (Papenbrock and Grimm, 2001), the MEP pathway is subject to multiple levels of regulatory controls. All transcripts of the MEP pathway genes are modulated in response to plant development. In addition, this work has demonstrated that the MEP pathway is regulated posttranscriptionally. Thus, the final activity of this pathway will probably reflect the integration of different regulatory mechanisms, such as the ones determined here and others yet to be identified. This shows the complex regulatory network that modulates this central biosynthetic pathway in plants.

**METHODS**

**Plant Material and Growth Conditions**

*Arabidopsis thaliana* Heyhn. wild-type ecotypes Ler, Columbia (Col-0), and Wassilewskija plants were used in this study. For experiments involving plants grown under sterile conditions, seeds were surface-sterilized and plated on germination (MS) medium containing 1× Murashige and Skoog basal salts (Gibco BRL, Grand Island, NY), 1% (w/v) sucrose, 1× Gamborg’s vitamin solution (Sigma-Aldrich, St. Louis, MO), and 0.05% (w/v) Mes [2-(N-morpholino) ethanesulfonic acid], solidified with 0.8% (w/v) phytoagar (Gibco BRL). Seedlings were grown under a 16-h light period at 230 μE/m²/s in growth chambers. Seeds were incubated at 4°C for 4 d to break dormancy before germination. Adult plants were grown in Metro-Mix 200 (Grace Sierra, Milpitas, CA). The clb4-1, clb5-1, and clb6-1 mutants are in Ler ecotype (Gutiérrez-Navá et al., 2004), whereas clb-1 is in Wassilewskija (Mandel et al., 1996) and the chl-1-2 is in Col-1 ecotype. Pigmentation mutant line 4036 (accession number AB009053) was generously provided by Gregory Budziszewski (Budziszewski et al., 2001), and SALK insertion lines 030640 and 002470 and the chlorina chl-2 mutant (CS3120) were obtained from the ABRC (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.html). Fosmidomycin treatment was performed following previous reports (Rojdríguez-Concepción et al., 2001; Okada et al., 2002; Laule et al., 2003). Wild-type Ler seedlings were grown for 8 d on filter paper disks saturated with MS liquid media. For treatment, the media was replaced by either...
MS (Fos –) or MS supplemented with 100 μM fosmidomycin (Fos +) (provided by Shinjiro Yamaguchi, Hiroyuki Kasahara, and Yuji Kamiya from the Plant Science Research Center RIKEN) and incubated for the indicated periods. Treatment with the herbicide norflurazon [SAN 9789, 4-chloro-S-(methylamino)-2-(a,a,a-trifluoro-m-toly)-3(2H)-pyridazinone] was performed by growing wild-type plants in MS media or MS with 5 μM norflurazon until the emergence of the first true leaves under a 16-h light cycle at 230 μE/m²/s.

**Pigment Determination**

Total carotenoids and chlorophylls were obtained from frozen tissue. Pigments were extracted during 5 h at room temperature with a methanol:dicloromethane mixture (2:1, v/v) in glass vials protected from light. Pigment levels were estimated in a Beckman DU®650 spectrophotometer at 663 nm (chlrophyll a), at 645 nm (chlrophyll b) (Arnon, 1949), and at 450 nm (carotenoids) (Davis, 1976).

**Gene Isolation**

Arabidopsis *ISPH* cDNA was amplified by RT-PCR using total RNA from 12-d-old wild-type Ler plants grown on MS media. The complete open reading frame (from ATG to TGA) of the *ISPH* gene was obtained using the oligonucleotides 5′-CTCTCATGGCTTGTTG-3′ (upper primer) and 5′-GAATCAAGGGCGTGC-3′ (lower primer). The resulting PCR product was cloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA) and checked by restriction enzyme analysis and sequencing (Medigenomix Eurofins Scientific Group, Martinsried, Germany). The genomic clone of the *ISPH* mutant gene was recovered from total DNA by standard PCR using the same set of primers.

**Mutant Complementation**

The complete cDNA of the *ISPH* gene was subcloned in the sense orientation into the pETG108 binary vector (http://ag.arizona.edu) between the CaMV 35S promoter and the nos 3′ terminator. The ISPH cDNA vector was transferred to the *Agrobacterium tumefaciens* strain LBA4404 (Matzke and Matzke, 1986) and used to transform heterozygote *cb6-1* mutant by vacuum infiltration (Bechtold et al., 1993). Hygromycin-B T0 resistant (50 μg/mL) transgenic lines were selected to obtain T1 plants for further analysis. Homozygous plants were identified by a 100% segregation of resistant plants in subsequent generations of 10 independent transgenic lines. The presence of the transgene in a mutant background was verified at the molecular level by RT-PCR using total mRNA from different transgenic plants. To corroborate the molecular identity of the transgenic plants, two specific 5′ primers were used: one (P35S) specific for the CaMV 35S promoter (5′-CCACACAGTCTTTCAAGC-3′) and the other (PISPH) for the *ISPH* gene promoter (5′-GTTTGTGCTTTGCTG-3′). A common 3′ primer to the *ISPH* coding region (5′-TCAAGCCAGCTG-3′ and 5′-GAA-CCAACCACGTCTTCAAAGC-3′) and the other (PISPH) for the *ISPH* gene was obtained using standard PCR with the appropriate pGEX vectors (Amersham Biosciences). The integrity of the chimeric genes was verified by direct sequencing (Medigenomix Eurofins Scientific Group). The IPTG-induced proteins were purified from *Escherichia coli* crude extracts by affinity chromatography using Glutathione Sepharose 4B resin (Amersham Biosciences) according to the protocol of the provider. For antibody generation against these three fusion proteins, 50 μg of purified GST-CMS, GST-CMK, and GST-MCS proteins in 1 mL of PBS (140 mM NaCl, 2.8 mM NaH₂PO₄, and 7.2 mM Na₂HPO₄, pH 7.4) and complete Freund’s adjuvant (Sigma-Aldrich) were subcutaneously injected as 1:1 (v:v) emulsion in 8-week-old female New Zealand rabbits. Six additional injections (50 μg each) with incomplete Freund’s adjuvant (Sigma-Aldrich) were administered at 10-d intervals starting 15 d after the initial injection. The serum of each rabbit was collected 3 d after the last injection and the titer determined for each protein. The generation of polyclonal antibodies against DXR and HDR was performed in rabbits using recombinant pQE:DXR and pQE:HDR proteins. The HDS antibody was raised against a synthetic peptide that was conjugated with keyhole limpet hemocyanin and used as an antigen to immunize rabbits. DXR, HDR, and HDS antibodies were kindly provided by Shinjiro Yamaguchi, Hiroyuki Kasahara, and Yuji Kamiya from the Plant Science Research Center RIKEN.

The specificity of the antibodies and the identity for each MEP protein was confirmed by the estimated molecular weight and by the absence of these bands in the corresponding preimmune sera (data not shown). Final confirmation was done by corroborating the absence of each protein in their corresponding mutants, *dxr* (line 4036, accession number AB009053) (Budziszewski et al., 2001), *ispD* (Salk, 030640/ISP5) and *ispF* (Salk, 002470) obtained from the Salk collection (http://signal.salk.edu), *cb6-1* (Gutiérrez-Nava et al., 2004), and *cb6-7* (this work). In the case of CMK, this confirmation was done by comparison of the protein
levels from antisense or overexpressing Arabidopsis transgenic lines generated in our laboratory (data not shown).

Protein Gel Blot Analysis

Total protein samples were obtained from frozen seedlings ground in liquid nitrogen and thawed in SDS sample buffer (0.125 M Tris-Cl, pH 6.8, 20% [v/v] glycerol, 4% [w/v] SDS, and 2% [v/v] 2-mercaptoethanol). The protein concentration was determined with Bradford reagent (Bio-Rad, Hercules, CA) using BSA as a standard and then separated by SDS-PAGE. To have comparable conditions in our protein analysis, 10 μg of total protein were used in all the protein analyses performed in this study. To verify equal protein loading, a parallel gel was run and stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich). The proteins were transferred onto nitrocellulose (Hybond C; Amersham-Pharmacia Biotech, Buckinghamshire, UK) by electroblotting for 1 h at 200 mA in 25 mM Tris, 0.2 M glycine, and 20% (w/v) methanol. Immunodetection was performed using a 1:1000 dilution of the polyclonal antibodies against DXS (Estévez et al., 2000), 1:1500 for DXR, CMK, MCS, HDS, and HDR, and 1:2500 for CMS. Optimization of each antibody dilution condition was established by demonstrating that detection was done under a linear range, using different protein concentrations (20, 10, 5, and 2.5 μg) of the same extracts used in the analyses (Figure 4C). Corroboration of linearity in two representative mutants (cis6-1 and ispD) and developmental stages was done following the same strategy (Figure 4D; see Supplemental Figures 1A and 1B online). An anti-rabbit immunoglobulin alkaline phosphatase conjugate was used as a secondary antibody (Zymed Laboratories, San Francisco, CA) and was detected using the BCIP/NBT substrate kit (Zymed Laboratories). Bands were quantified using NIH Image software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Phylogenetic Trees

Sequences for analyzed proteins were retrieved from the National Center for Biotechnology Information Web site (http://www.ncbi.nlm.nih.gov) after a similarity search performed using the BLAST algorithm (Wisconsin Package version 10.0; Genetics Computer Group, Madison, WI) (Altschul et al., 1990). Amino acid sequences were aligned using the ClustalW algorithm (http://www2.ebi.ac.uk/clustalw). Phylogenetic relationships were constructed by parsimony using the PAUP*SEARCH algorithm of the GCG package (Wisconsin Package Version 10.0).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY883838.

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Characterization of the Arabidopsis clb6 Mutant Illustrates the Importance of Posttranscriptional Regulation of the Methyl-d-Erythritol 4-Phosphate Pathway

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