THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 25, Issue of June 22, pp. 22901–22909, 2001

*Printed in U.S.A.*

# 1-Deoxy-D-xylulose-5-phosphate Synthase, a Limiting Enzyme for Plastidic Isoprenoid Biosynthesis in Plants*

Received for publication, January 30, 2001, and in revised form, March 19, 2001
Published, JBC Papers in Press, March 22, 2001, DOI 10.1074/jbc.M100854200

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**The initial step of the plastidic 2C-methyl-D-erythritol 4-phosphate (MEP) pathway that produces isopentenyl diphosphate is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase. To investigate whether or not 1-deoxy-D-xylulose-5-phosphate synthase catalyzes a limiting step in the MEP pathway in plants, we produced transgenic *Arabidopsis* plants that over- or underexpress this enzyme. Compared with non-transgenic wild-type plants, the transgenic plants accumulate different levels of various isoprenoids such as chlorophylls, tocopherols, carotenoids, abscisic acid, and gibberellins. Phenotypically, the transgenic plants had slight alterations in growth and germination rates. Because the levels of several plastidic isoprenoids correlate with changes in 1-deoxy-D-xylulose-5-phosphate synthase levels, we conclude that this enzyme catalyzes one of the rate-limiting steps of the MEP biosynthetic pathway. Furthermore, since the product of the MEP pathway is isopentenyl diphosphate, our results suggest that in plastids the pool of isopentenyl diphosphate is limiting to isprenoid production.**

Isoprenoids are a group of biologically active molecules that number in the tens of thousands. Members of this diverse group of natural products are found in all organisms. In higher plants, isoprenoids participate in a wide variety of biological functions such as photosynthesis, respiration, growth, cell cycle control, plant defense, and adaptation to environmental conditions. Specific examples include photosynthetic pigments (chlorophylls and carotenoids), hormones (abscisic acid (ABA),[1] gibberellins (GA), cytokinins, and brassinosteroids), a side chain of the electron transporter (plastiquinone), structural components of membranes (phytosterols), and antimicrobial agents (phytoalexins). Beyond these plant-specific functions, many plant isoprenoids have been shown to have industrial and medical importance. The plant-produced isoprenoids β-carotene (provitamin A) and α-tocopherol (vitamin E) are both basic nutrients required for the maintenance of human health (1–4). Another plant-produced isoprenoid, Taxol, is used as a chemotherapeutic agent in the treatment of cancer (5). Moreover, drugs that block isoprenoid production in *Plasmodium falciparum* are being evaluated as anti-malarial agents (6). Industrial uses of isoprenoids include products such as colorants, fragrances, and flavorings (7). A detailed understanding of isoprenoid biosynthetic pathways and their regulation is essential to fully exploit these and future uses of isoprenoids.

Isoprenoids are derived by consecutive condensations of five-carbon precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). From these common precursors, the biosynthetic pathways of the various isoprenoids diverge. The work of several groups has demonstrated that in plants two distinct pathways synthesize IPP (Fig. 1). The acetate/mevalonate (MVA) pathway (8, 9), which is shared with animals and fungi (10), occurs in the cytoplasm where sesquiterpenes ($C_{15}$) and triterpenes ($C_{30}$) such as sterols are produced (Fig. 1). The more recently identified MEP pathway (named for the first committed molecule in the pathway, 2C-methyl-D-erythritol 4-phosphate) occurs in plastids and is also found in protozoa, most bacteria, and algae (6, 11–16). This plastidic pathway produces IPP that is used for the biosynthesis of isoprene, monoterpenes ($C_{10}$), diterpenes ($C_{20}$), carotenoids, plastiquinones, and phytol conjugates such as chlorophylls and tocopherols (16–18). Although there is evidence that some limited exchange occurs in plants between the cytoplasmic and plastidic pools of IPP, each pathway appears to produce unique isoprenoids (15, 19).

Whereas all of the genes involved in the MVA pathway have been identified, only the first genes of the plant MEP pathway have been published (Fig. 1). The first step in the MEP pathway involves a transketolase-type condensation reaction of pyruvate and glyceraldehyde 3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (DXP). This reaction is catalyzed by DXP synthase (DXS). Genes encoding DXS have been cloned and characterized in *Escherichia coli* (20, 21), *Mentha* × *piperita* (22), *Capsicum annuum* (23), *Synechococcus leopoliensis* (24), *Lycopersicon esculentum* (25), *Streptomyces* (26), and *Arabidopsis thaliana* (27, 28). In plants, the DXP produced by this reaction is utilized in plastidic IPP biosynthesis as well as in the production of thiamin and pyridoxol (29, 30). The subsequent steps of the MEP pathway have been shown to be specific for IPP production, and the genes coding for the next four steps have been identified in both bacteria and plants (Fig. 1). The first step specific for IPP production is the transformation of DXP to MEP by the enzyme DXP reductoisomerase (DXR) (6, 31–34). MEP is subsequently converted into 2C-methyl-D-erythritol 2,4-cyclodiphosphate by the consecutive activities of three independent enzymes as shown in Fig. 1 (35–39). The

* This research was supported by Consejo Nacional de Ciencia y Tecnología Grant 31791-N, Dirección General de Asuntos del Personal Academico Grant IN205697, and by BASF. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ABA, abscisic acid; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MVA, acetate/mevalonate; MEP, 2C-methyl-D-erythritol 4-phosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; GA, gibberellins; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase-PCR; MES, 4-morpholineethanesulfonic acid.

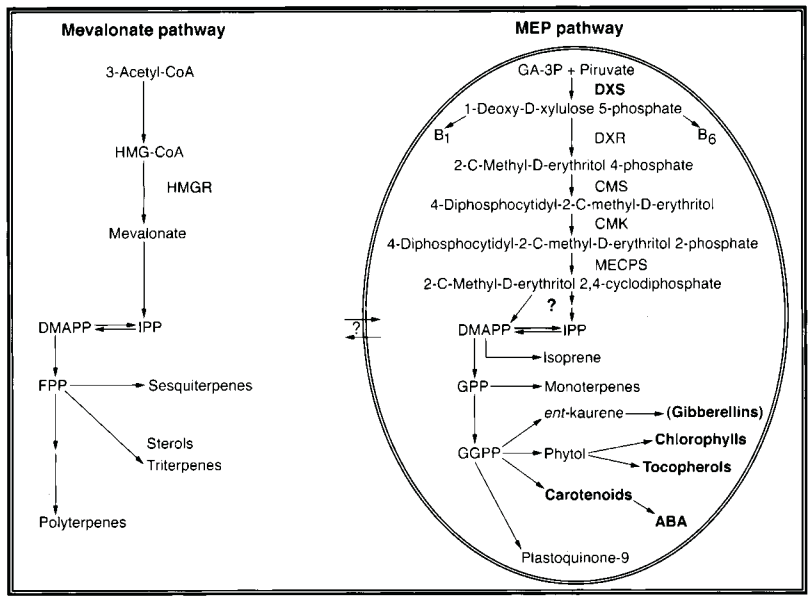


FIG. 1. **IPP and isoprenoid biosynthetic pathways in plants.** Diagramatic representation of the cytoplasmic mevalonate and plastidic MEP pathways is shown. *Arrows* in and out of the plastid indicate the postulated limited exchange between the cytoplasmic and plastidic IPP pools. *?* denotes unidentified steps in the MEP pathway. (Gibberellins) indicates that the precursors for biosynthesis of gibberellins are formed in plastids. *HMG-CoA,* hydroxymethylglutaryl-CoA; *HMGR,* hydroxymethylglutaryl-CoA reductase; *DMAPP,* dimethylallyl diphosphate; *IPP,* isopentenyl diphosphate; *GA-3P,* glyceraldehyde 3-phosphate; $B_1$, thiamin; $B_6$, pyridoxol; *CMS,* 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; *CMK,* 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; *MECPS,* 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; *GPP,* geranyl diphosphate; *GGPP,* geranylgeranyl diphosphate; *FPP,* farnesyl diphosphate; ABA, abscisic acid.

final steps leading to IPP remain unknown.

Identifying the different enzymes involved in the synthesis of IPP is only part of the work necessary to characterize such a complex biosynthetic pathway. Another fundamental aspect is the identification of the limiting steps in the pathway. Large changes in the level of a non-limiting enzyme can be made with little or no effect on the quantity of the final product; therefore, one of the most fruitful strategies for altering isoprenoid content will be those that focus on the rate-limiting enzymes (40). In the case of the MEP pathway, recent studies in bacteria (24, 26, 34) and tomato (25) suggest that DXS is a rate-limiting enzyme.

In this article we report on the effects of altering DXS levels, the first gene in plastidic isoprenoid synthesis in plants. Our previous work demonstrated that, in *A. thaliana*, DXS is encoded by the *CLA1* gene (28). In order to explore the participation of DXS in plastidic isoprenoid synthesis in plants, the enzyme levels were increased or decreased in *Arabidopsis* plants. Analysis of several transgenic lines showed that plants overexpressing DXS had increased levels of isoprenoids such as chlorophylls, tocopherols, carotenoids, ABA, and GA. Moreover, plants with suppressed levels of DXS had decreased amounts of all of these products. The fact that alterations in DXS levels lead to changes of various isoprenoid end products demonstrates that DXS is one of the limiting steps in the production of plastidic IPP and, therefore, of isoprenoids in higher plants. Each group of plastidic isoprenoids has a distinct biosynthetic pathway that could be manipulated; however, our findings indicate that the output of these pathways could be restricted by the amount of IPP that is produced in the plastids.

## EXPERIMENTAL PROCEDURES

*Plant Material and Growth Conditions—A. thaliana* plants were grown on Metro-Mix 200 soil (Grace Sierra, Milpitas, CA) in a controlled growth chamber (24 °C, 16:8 h light:dark photoperiod). Seeds under sterile conditions were surface-sterilized and plated on germination medium (GM) containing 1× Murashige and Skoog basal salt mixture supplemented with B5 vitamins, 0.05% MES, 1% sucrose, and 0.8% phytoagar. As *cla1-1* seed stocks are heterozygous, seeds were initially germinated on GM, and the albino homozygous mutant plants were selected for all of the subsequent analyses. To select heterozygous plants, seeds were germinated on kanamycin GM media. Plate-grown, 15-day-old plants were used for the Northern blots, Western blots, and isoprenoid quantifications.

The analysis of seed dormancy was performed using seeds that were never exposed to cold treatment. Germination time was taken at the emergence of the root.

*Arabidopsis Transformation—CLA1* cDNA consisting of either the ATG to the stop codon in a sense or 650 base pairs of the 3′ region of the cDNA for the antisense orientation was inserted into the binary vector pBin19 (41) containing the neomycin phosphotransferase II gene as a selectable marker (Fig. 2*A*). For both constructs the *CLA1* cDNA is under the control of the cauliflower mosaic virus 35S promoter. The constructs were introduced into *Arabidopsis* via an *Agrobacterium tumefaciens*-mediated root explant transformation (42). Transgenic plants were identified by their ability to develop leaves in the presence of 50 $\mu$g ml$^{-1}$ kanamycin. They were then transferred to soil to obtain seeds for the subsequent generations. Homozygous plants were identified by a 100% segregation of kanamycin-resistant plants in the progeny.

*Northern Blot Analysis*—Total RNA was isolated from frozen tissue essentially as described (43). The RNA was fractionated by electrophoresis on 1.2% agarose gels and transferred onto Hybond N$^+$ nylon membrane (Amersham Pharmacia Biotech). Hybridizations and washes were carried out at high stringency according to standard procedures using $^{32}$P-radiolabeled probes (44).

*Western Blot Analysis*—Total protein samples were obtained by snap-freezing the plants in liquid nitrogen and grinding them in the presence of SDS sample buffer (0.125 M Tris-Cl, pH 6.8, 20% v/v glycerol, 4% w/v SDS, 2% v/v 2-mercaptoethanol). Protein samples were quantified with Bradford reagent (Bio-Rad) and then separated by SDS-polyacrylamide gel electrophoresis. To verify equal protein loading, a parallel gel was run and stained with Coomassie Brilliant Blue R-250. The proteins were transferred onto nitrocellulose (Hybond C, Amersham Pharmacia Biotech) 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 1:1,000 dilution of the polyclonal antibody raised against a GST-CLA1 fusion protein (28). An anti-mouse immunoglobulin horseradish peroxidase conjugate was used as a secondary antibody (Amersham Pharmacia Biotech), and detection was carried out with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). Bands were quantified using NIH Image software (Wayne Rasband, National Institutes of Health, Bethesda).

*Determination of Chlorophyll and Carotenoid Content*—Determination of total carotenoids and chlorophylls was conducted as described elsewhere (45). Extracts were obtained in 100% acetone from 100 mg of fresh tissue from 15-day-old *Arabidopsis* seedlings. Spectrophotometric quantification was carried out in a Beckman DU$^R$650 spectrophotometer.

*Determination of Tocopherol Content*—Extracts were obtained from 500 mg of 15-day-old plants, frozen in liquid $N_2$, and homogenized. To the pulverized material a mixture of chloroform/methanol (1:1 v/v) was added, and the solution was transferred into two 4-ml centrifuge flasks, followed by vortexing for 15 min at 4 °C. The homogenate was centrifuged, and the supernatant was transferred to a 25-ml measuring flask. The pellet was re-extracted with 2 ml of the chloroform/methanol mixture and dried completely in a rotary evaporator at 60 °C. To remove residual water, 2 ml of ethanol were added and then evaporated completely. The sample was resuspended in 1 ml of heptane, filtered, and loaded onto a Lichrosorb Si 60 column (5 $\mu$m 250 × 4 mm; Merck) with

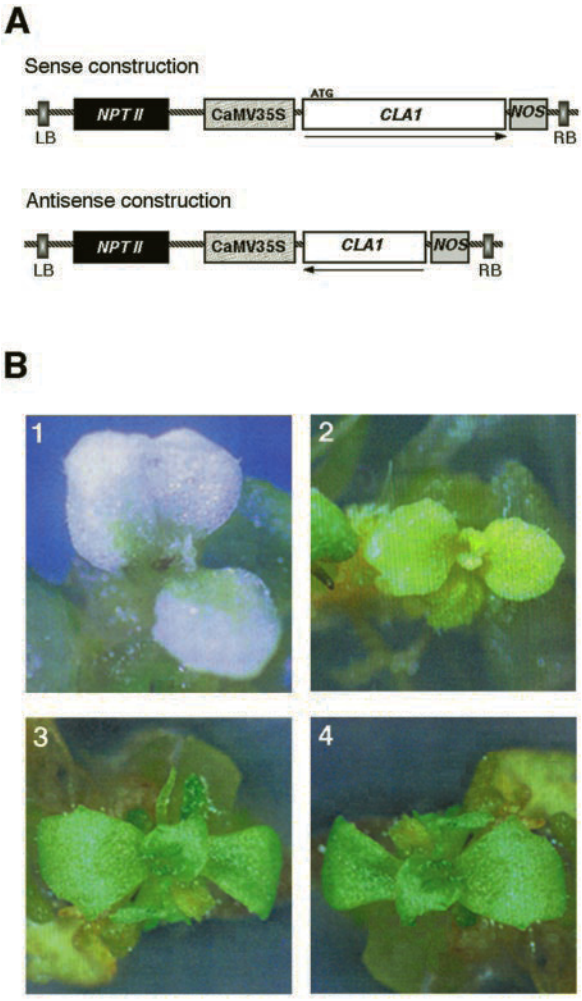


FIG. 2. **CLA1 constructs and their phenotypes.** *A,* diagram of the constructs used in plant transformation. The sense construct contains 2.2 kilobase pairs of the *CLA1* cDNA including the complete coding region from the ATG to the stop codon. The antisense construct contains 0.65 kilobase pairs of the 3′ end of the *CLA1* cDNA. *LB, left* T-DNA border; *RB, right* T-DNA border; cauliflower mosnic virus (CaMV) 35S promoter; *NOS*, polyadenylation signal from nopaline synthase; *NPT II*, neomycin phophotransferase II. *B*, representative phenotypes obtained in the antisense (1–3) and sense transgenic plants (4).

a mobile phase of heptane and dioxane (97:3) and a flow of 1 ml/min. The injected volume was 10 $\mu$l at room temperature. Peaks were detected using a fluorescent detector with emission at 330 nm and excitation at 295 nm.

*Determination of ABA Content*—To quantify the ABA content of the transgenic and wild-type plants, 10 mg of fresh 15-day-old tissue were homogenized in 1 ml of ABA extraction buffer (10 mM HCl, 1% polyvinilpoly-pyrolidene in methanol) and incubated overnight at 4 °C with constant shaking. The supernatant was collected and neutralized with 15 $\mu$l of 1 M NaOH as described (46). ABA was quantified with a Phytodetek-ABA-kit (Agdia Inc., Elkhart, IN) using the protocol provided but with the addition of TBS and 0.1% gelatin to all of the samples.

*Determination of GA4 Expression*—Total RNA was isolated using the procedure of Logemann *et al*. (43). The cDNAs were synthesized from 5 $\mu$g of total RNA by an oligo(dT)-primed reverse transcription using M-MLV reverse transcriptase (Life Technologies, Inc.). An aliquot of the first strand cDNA (10%) was used as template in a standard 50 $\mu$l of PCR (with 0.25 mM dNTPs and 100 ng for each primer). Each PCR was checked to ensure that it was in the linear range by removal of a small sample (5 $\mu$l) of the PCR at 18, 21, 24, and 30 cycles during the PCR program (1 min each at 94, 55, and 72 °C). The primers used and the corresponding products are as follows: 5′-CGATTTCCGTAAACTTTGGC-3′ and 5′-ATCCATTGGATAGGATGTGG-3′ were used to amplify a 398-base pair fragment of *GA4* cDNA; and 5′-TCCCAGAATCGCTAAGATTGCC-3′ and 5′-CCTTTCCCTTAAGCTCTG-3′ were used to amplify a 478-base pair fragment of *APT1* cDNA. PCR products were separated by electrophoresis, blotted, and hybridized using standard techniques ([$^{32}$P]dCTP-labeled hybridization probes). RT-PCR products were quantified using NIH Image software (Wayne Rasband, National Institutes of Health, Bethesda).

## RESULTS

*Production of Transgenic Arabidopsis*—DXS catalyzes the first step of the MEP pathway and has been proposed to be a limiting step for the production of IPP in bacteria and plants (24–26, 34). In earlier work we identified the *CLA1* gene as coding for the functional *Arabidopsis* version of DXS (28). To test the role of DXS in regulating the levels of plastidic isoprenoids in plants, constructs containing the *CLA1* open reading frame (27) under the control of the cauliflower mosaic virus (CaMV) 35S promoter were constructed in either a sense or an antisense orientation with a duplicated enhancer region at the 5′ end (Fig. 2*A*). These constructs were inserted into pBin19, a T-DNA derived vector, and introduced into *Arabidopsis* via an *A. tumefaciens*-mediated root explant transformation (42). Transgenic $T_0$ lines were selected on kanamycin-containing medium. Sense plants showed a green phenotype, similar to wild-type plants, whereas antisense plants exhibited a range of phenotypes from albino to pale-green and fully green (Fig. 2*B*). Such phenotypes are in agreement with the previously reported essential role of the *CLA1* gene in *Arabidopsis* plants (27). Since albino and pale-green $T_0$ plants do not survive long enough to produce seeds, only seeds from the green kanamycin-resistant $T_0$ transgenic plants could be rescued and grown to generate subsequent generations. Thus, we know that our antisense selection scheme is limited to moderate DXS suppression levels that permit viable plants. These $T_1$ plants were then self-propagated to obtain a homozygous transgenic $T_2$ population for each independently transformed line. Several of the $T_2$ lines were analyzed for DXS levels (data not shown), and a few of those lines with the greatest differences in DXS levels were selected for further evaluation.

*Molecular Characterization of the Transgenic Lines*—In order to compare mRNA and protein steady-state levels for the *Arabidopsis* DXS (*CLA1* gene), 15-day-old seedlings from sense (S-2, S-17, S-14, and S-7), antisense (A-6 and A-18), wild-type plants, as well as homozygous *cla1-1* and heterozygous *CLA1*/*cla1* mutants (27) were analyzed by Northern and Western blots. As shown in Fig. 3*A*, the sense plants of the S-2, S-14, and S-17 lines had increased expression levels of the *DXS* mRNA compared with wild-type plants. Some of the sense plants such as the S-7 line contained very low levels of the *DXS* mRNA compared with wild-type plants. This may be attributable to gene silencing, a phenomenon commonly observed in transgenic plants (47). The Northern blot analysis also showed that the antisense plants of the A-6 and A-18 lines have reduced accumulation of the *DXS* transcript compared with wild-type plants, as do the *CLA1*/*cla1* heterozygous plants (Fig. 3*A*). Consistent with our data reported previously (28), no *DXS* mRNA expression was detectable in the *cla1-1* mutant. Notable in the Northern blot is the slightly smaller size of the mRNA bands in the overexpressing lines (S-2, S-14, and S-17). This is likely due to the smaller 3′- and 5′-untranslated regions of the sense constructs, which cause the mRNA of the sense constructs to be slightly smaller than wild-type mRNA (Fig. 3*A*).

Western blot analysis was then performed to check for the accumulation of the DXS protein in all of the selected plants (Fig. 3*B*). Transgenic plants expressing high levels of the *DXS* mRNA such as S-2, S-17, and S-14 also accumulated high levels of the DXS protein compared with wild-type levels. In contrast, the lines expressing low levels of the *DXS* mRNA (S-7, A-6, A-18, and *CLA1*/*cla1*) accumulated low levels of the DXS protein compared with the wild-type levels. As published previ-

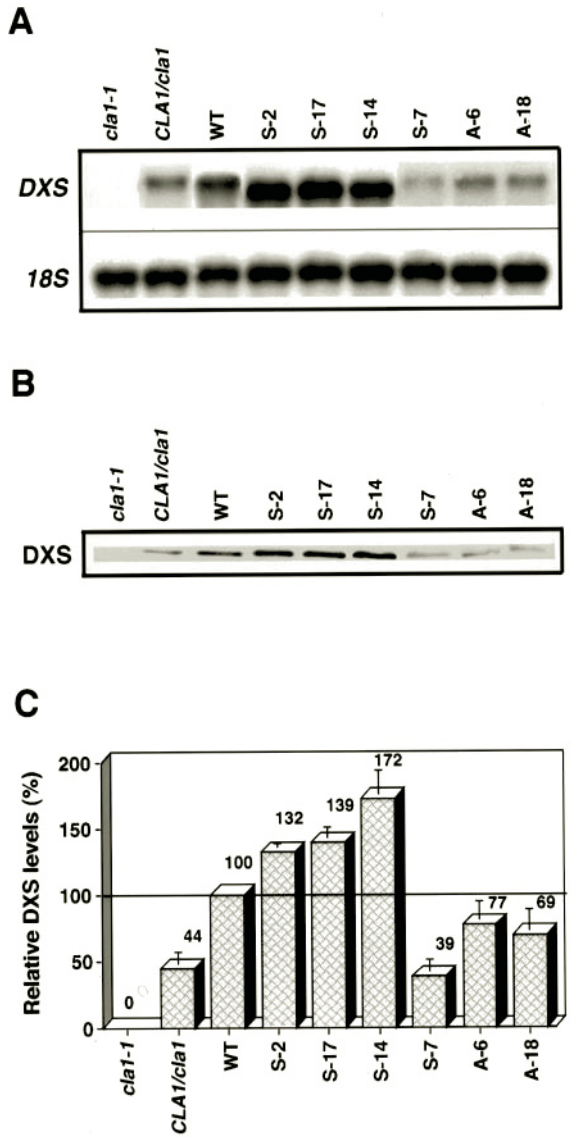


FIG. 3. **Expression of *DXS* in transgenic and wild-type plants.** *A*, RNA gel blot analysis. 5 $\mu$g of total RNA was loaded in each lane isolated from 15-day-old seedling from DXS mutant (*cla1-1*), heterozygous (*CLA1/cla1*), wild type (*WT*), overexpressing (S2, S17, and S14), and suppressed (S7, A6, and A18) plants. The blot was hybridized with *CLA1* and 18 S rRNA probes. *B*, Western blot analysis. 15 $\mu$g of total protein isolated from 15-day-old seedlings of each plant were loaded per lane and analyzed by SDS-polyacrylamide gel electrophoresis. The blot was probed with a DXS-specific antibody. *C*, the intensity of the bands from the Western blot was determined quantitatively by densitometric analysis. *Columns* represent means as a percentage of wild-type levels of three individual experiments. *Bars* represent standard deviation.

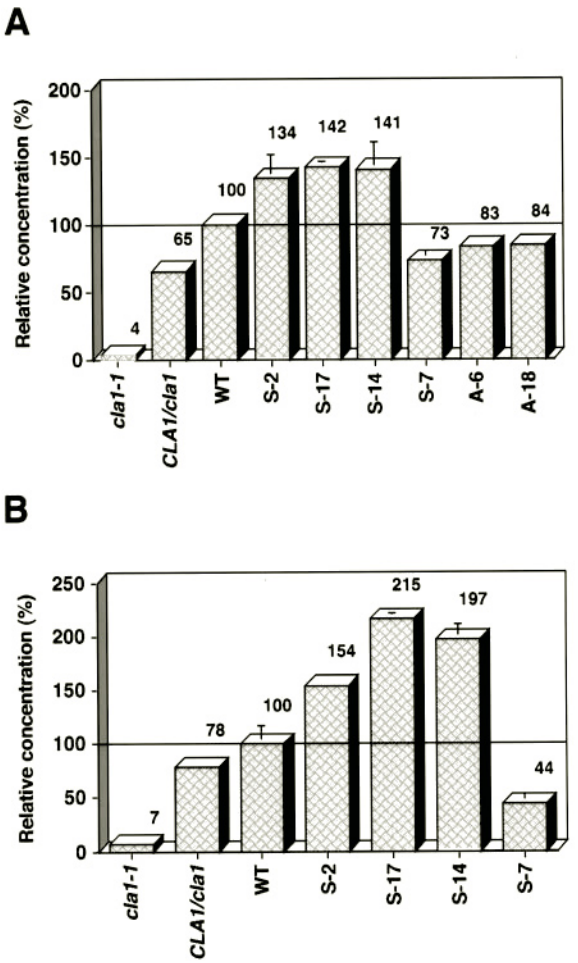


FIG. 4. **Total chlorophyll and $\alpha$-tocopherol content in transgenic and wild-type plants.** *A*, total chlorophyll content; *B*, $\alpha$-tocopherol content extracted from 15-day-old seedling. Each *column* represents the mean of three independent experiments expressed as a percentage relative to wild-type levels, which are marked as 100%. Standard deviation is shown by *bars* (sometimes smaller that the scale).

ously for the *cla1-1* mutant (28), no DXS protein could be detected either. The densitometric quantification of the DXS protein level (Fig. 3*C*) showed that by a comparison to wild-type plants, lines S-2, S-17, and S-14 are overexpressing the DXS protein ranging from 132 to 172%. On the other hand, lines S-7, A-6, A-18, and *CLA1/cla1* contain between 38 and 77% of wild-type DXS protein levels, and *cla1-1* mutants show no detectable DXS protein.

*Effects of Altered Expression of DXS on Plastidic Isoprenoid Content*—Many isoprenoids are formed in plastids via the IPP produced by the MEP pathway. In order to determine the impact of altered DXS levels on isoprenoid content, the quantities of plastidic isoprenoids such as chlorophylls, tocopherols, carotenoids, ABA, and GA were measured in the selected transgenic lines and compared with the levels found in wild-type plants. These isoprenoids were chosen, in part, because they are formed from three pathways that diverge from the common plastidic IPP precursor (15, 19).

*Total Chlorophyll and Tocopherol Content*—Chlorophylls and tocopherols are two common plant isoprenoids that are formed, via IPP, from phytol. Chlorophyll consists of two moieties. Chorophyllide, which is not an isoprenoid, is formed from the precursor molecule 5-aminolevulinate. The other moiety is the isoprenoid, phytol. As shown in Fig. 4*A*, in the DXS-overexpressing plants (S-2, S-14 and S-17) the total chlorophyll content increased with chlorophyll levels ranging from 134 to 142% of the wild-type levels ($p < 0.05$). In plants with suppressed levels of DXS (S-7, A-6, A-18, and *Cla*I/*cla1*), it was observed that the total chlorophyll content decreased between 65 and 84% of wild-type levels ($p < 0.003$). Additionally, in the *cla1-1* mutant plants, the total chlorophyll levels were only 4% of the wild-type levels, which agreed with the previously published (27) measurements for this mutant.

Phytol is also a precursor for the synthesis of tocopherols, and it is directed into the tocopherol-synthesizing pathway by condensation with homogentisic acid derived from the shikimate pathway (48). It is well established that several methylations and a cyclization step of the quinol intermediate lead to $\alpha-$tocopherol, the major form of vitamin E (48). Plants with altered levels of DXS have changes in their $\alpha$-tocopherol content (Fig. 4*B*). Similar to what we found with chlorophyll con-

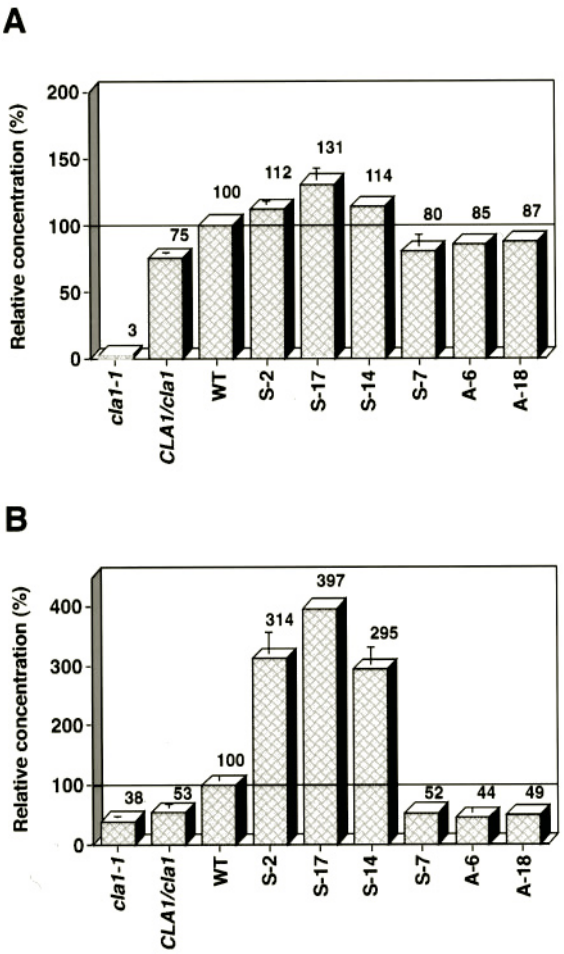


FIG. 5. **Carotenoids and abscisic acid content in transgenic and wild-type plants.** *A*, total carotenoid content; *B*, ABA content from 15-day-old seedling. In both cases *columns* represent means as a percentage relative to wild-type levels (100%). *Bars* represent standard deviation of three independent replicates (sometimes smaller that the scale).

tent, the transgenic plants with higher levels of the DXS protein (S-2, S-14, and S-17) have from 154 to 215% of the wild-type levels of $\alpha$-tocopherol ($p < 0.01$), whereas plants with reduced levels of DXS (S-7 and *CLA1*/*cla1*) have 43 and 78% of wild-type levels, respectively ($p < 0.01$). In this case the *cla1-1* mutant also produces very low levels of $\alpha$-tocopherol at only 7% of the wild-type levels. Thus, modulations of DXS levels cause changes in the quantity of these two phytol-derived compounds, chlorophyll and $\alpha$-tocopherol.

*Carotenoids and Abscisic Acid Content*—Carotenoids are formed in plastids via phytoene, a $C_{40}$ precursor molecule (49). Experimental evidence has clearly demonstrated that carotenoids are one of the major products derived from the MEP pathway (15). Thus, we were interested in quantifying these compounds in our transgenic plants. As *Arabidopsis* plants do not contain chromoplast-accumulating tissues, our analysis was limited to the quantification of the carotenoids present in photosynthetic tissues. We found that altered levels of DXS also result in changes in the levels of total carotenoid content in the transgenic plants. Plants that are overexpressing DXS, such as S-2, S-14, and S-17, show increases in total carotenoids from between 112 and 131% relative to the wild-type levels ($p < 0.02$) (Fig. 5*A*). Plants with suppressed levels of DXS (S-7, A-6, A-18, and *CL A1*/*cla1*) contain less total carotenoids, between 75 and 87% of wild-type levels ($p < 0.05$). As reported (27), the *cla1-1* mutant plants produced extremely low levels of carotenoids, with only 3% of wild-type levels.

It has been shown that a major part of ABA biosynthesis is carried out in the plastids from a $C_{40}$ carotenoid precursor (50). Thus, the level of DXS enzymatic activity might affect the levels of this hormone. In order to explore if ABA levels are dependent on changes in the levels of DXS, the endogenous ABA levels were measured in our transgenic plants. We observed that varying the quantity of DXS also affects ABA levels (Fig. 5*B*). The lines with more DXS, such as S-2, S-14, and S-17, accumulated substantially higher ABA levels (295–397%) compared with wild-type ($p < 0.0002$). On the other hand, in those transgenic plants with reduced DXS levels (S-7, A-6, A-18, and *CLA1*/*cla1*), the relative ABA content is reduced with 44–53% ($p < 0.01$) of the wild-type levels (Fig. 5*B*). In the *cla1-1* null mutant, there is a further reduction in the amount of ABA to 38% compared with wild-type plants.

These results demonstrate that a second pathway that branches from IPP is similarly affected by differences in DXS levels. The levels of carotenoids are increased or reduced in concert with the level of DXS. Additionally, altered DXS levels likewise affect the synthesis of the hormone ABA. It is of particular interest that the changes in ABA were greater than those observed for total carotenoids. As shown in Fig. 5, the increase in total carotenoids is more restricted than for ABA. It is known that the carotenoid biosynthetic pathway is highly regulated, thus it is possible that additional limiting reaction steps for specific carotenoids exist. For example lutein, which plays a central role in the photosynthetic apparatus, is derived from $\alpha$-carotene, whereas zeaxanthin, the direct precursor of ABA, is derived from $\beta$-carotene (49). It is possible that only specific carotenoid intermediates such as xanthoxin, a direct precursor of ABA, are more affected than others. As we have quantified total carotenoid content, an increase or decrease of a particular carotenoid might not be reflected in the total carotenoid levels in photosynthetic tissues. A similar situation has been reported with the reduction of phytone synthase using antisense RNA in tomato plants, where a drastic reduction (97%) of carotenoid accumulation was found in fruits without a noticeable effect in leaf tissue (51). Also there is the case of DXS overexpression in bacteria where a differential accumulation of carotenoid and ubiquinone-8 was observed (52). On the other hand, the capacity of various tissues to make and/or degrade carotenoids may differ substantially. It would be of particular interest to explore the impact of an increase of DXS activity on the biosynthesis of carotenoids in chromoplast-containing tissues in plants such as tomato, where DXS has been suggested to be a limiting enzyme (25).

*Gibberellins*—GA constitute a large family of diterpenes that act as plant hormones and are involved in many developmental processes. It is known that these compounds are formed by converting geranylgeranyl diphosphate to *ent*-kaurene and that these initial steps are carried out in plastids (53). Even though important advances have been made in the understanding of the GA biosynthetic pathway, the origin of these compounds is still uncertain. Although it is likely that GA are derived from the MEP pathway, until now this has not been conclusively proven (54). We decided to explore the effect of altered DXS expression on GA levels. Due to the wide range of active and inactive GA and the inherent difficulty in accurately measuring them, we used the level of the *GA4* gene as an indirect marker for the level of GA (55). It has been shown that transcription of the *GA4* gene is controlled by a negative feedback mechanism regulated by the amount of active GA; therefore, the relative level of the *GA4* gene has an inverse relationship to the amount of active GA present in the plant (55). RT-PCR experiments using specific primers for *GA4* and *APT1*

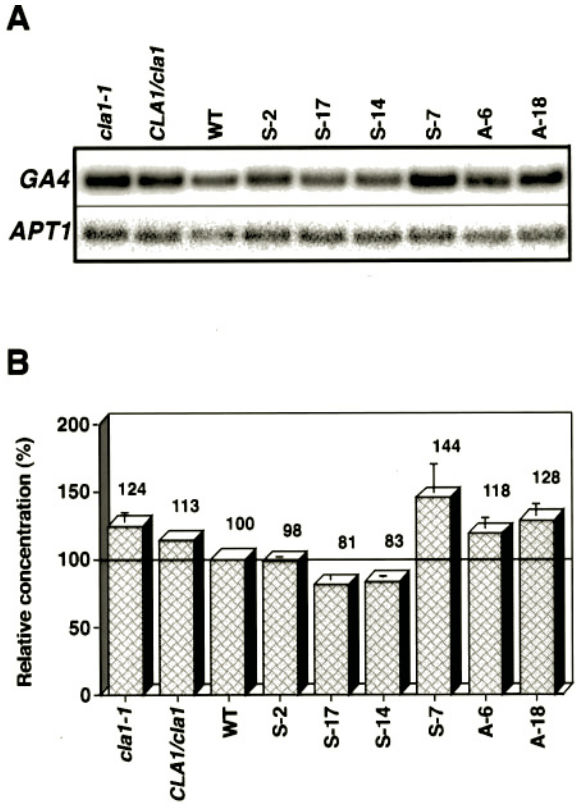


FIG. 6. **Expression of *GA4* in transgenic and wild-type plants.** *A*, representative gel from a RT-PCR expression analysis of the *GA4* and *APT1* (constitutive expressing control) transcripts in (*cla1-1*), heterozygous (*CLA1/cla1*), wild-type (*WT*), and overexpressing (S2, S17, and S14) and under-expressing (S7, A6, A18) plants. *B*, densitometric quantification of the GA4 transcript levels corrected by the APT1 transcript. Each *column* represents the GA4/APT1 ratio expressed as a percentage relative to the wild-type levels. *Bars* represent standard deviation of two independent experiments (sometimes smaller that the scale).

(a constitutively expressed control) showed that alteration of DXS levels caused changes in *GA4* transcript accumulation (Fig. 6*A*). As shown in Fig. 6*B*, for those transgenic plants accumulating higher DXS (S-2, S-14, and S-17), there was less *GA4* transcript detected, ranging from 81 to 98% of wild-type levels. On the other hand, the transgenic plants with lower levels of DXS (S-7, A-6, A-18, and *CLA1/cla1*), showed between 113 and 144% of the *GA4* transcript level compared with wild-type plants. The *cla1-1* null mutant also accumulated higher levels of GA4 transcript (124%) compared with wild-type plants. Because of the method used to quantify GA levels, the changes observed are not directly comparable to the changes in the other isoprenoids quantified in this work. But the general pattern found for the other isoprenoids remains true for GA. Raising the amount of DXS results in higher GA levels and lowering the amount of DXS lowered GA levels.

*Phenotypes of Transgenic Plants*—To determine if the observed changes in isoprenoid levels affect plant morphology, plants with wild-type, overexpressed, and suppressed levels of DXS were grown and their phenotypes compared. No appreciable difference in the general plant morphology was observed between our transgenic plants and the wild-type control, except for their size. In order to explore this phenotype more closely, the size of the transgenic plants was estimated in two developmental stages by the hypocotyl elongation at the seedling stage, 8 days old, and later, adult plants were measured for bolt length. We observed that at 8 days old (Fig. 7*A*), the wild-type plants had hypocotyl lengths averaging 3.8 cm, meanwhile the

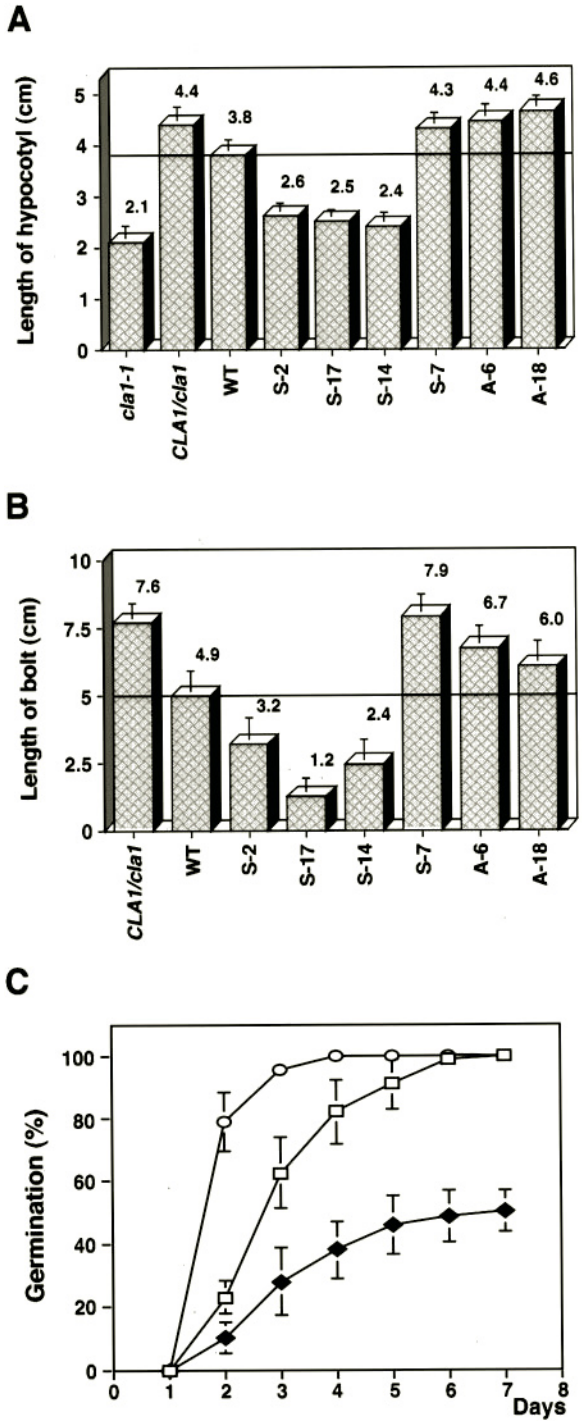


FIG. 7. **Phenotype analysis.** *A*, length of the hypocotyl of 8-day-old seedlings. The *horizontal line* represents the average size of wild-type plants. *Columns* represent the average size of 20 plants for each mutant, control, and transgenic line. *B*, bolt size of 26-day-old plants. Plants grown in soil were measured for the length of their bolt 26 days after imbibition. The *horizontal line* represents the size of the wild-type plants, and the *columns* are the mean of 15–20 plants for each line. The standard deviations in *A* and *B* are shown by *bars*. *C*, seed dormancy. Seeds from wild-type (*open squares*), ove-expressing S-17 (*close diamonds*), and suppressed S-7 (*open circles*) plants were germinated without cold treatment. Germination was scored when the radicle tip had fully emerged from the seed coat. These data represent the average of three independent experiments evaluating 100 seeds each; the *bars* represent the standard deviation.

DXS-overexpressing plant lines (S-2, S-14, and S-17) had shorter hypocotyls (2.4 to 2.6 cm, $p < 0.0001$), and the DXS suppressed plants (S-7, A-6, A-18, and *ClaI/cla1*) had longer

hypocotyls (4.3 to 4.6 cm, $p < 0.0001$). As expected, the albino *cla1-1* mutant plants were the smallest of all the plants measured with an average of 2.1 cm (Fig. 7*A*).

The effect of various DXS levels was also determined for the length of the bolt in 26-day-old plants grown in soil. As shown in Fig. 7*B* the observed pattern was similar to the hypocotyl measurements in that the DXS-overexpressing plants were shorter (1.2–3.2 cm, $p < 0.0002$) than wild-type plants (4.9 cm), and plants with suppressed levels of DXS were taller (6.0 to 7.9 cm, $p < 0.008$) than wild-type plants. However, these unexpected differential growth phenotypes of the transgenic plants are a transient condition because at 30 days post-germination all of the plant lines reached approximately the same size (data not shown).

Since ABA levels had the biggest relative change of all isoprenoids that were measured (Fig. 5*B*), we decided to further substantiate its biological effect in our transgenic plants. It is well known that ABA plays an important role in mediating responses to environmental stresses as well as in other developmental processes such as the establishment of seed dormancy (56). When grown under controlled conditions, none of our underexpressing transgenic plants showed apparent phenotypes related to ABA deficiency such as a wilty phenotype (57). Similarly, none of the plants with higher ABA levels showed symptoms that have been related to high ABA content in tomato plants such as overguttation (58). However, we observed that seeds from our transgenic plants germinated at slightly different rates than wild-type plants; thus we tested for changes in seed dormancy. Fig. 7*C* shows the germination rates for non-cold-treated seeds from wild type, a DXS-overexpressing line (S-17) containing almost 4 times more ABA than wild type, and a DXS-suppressed line (S-7) with about one half of wild-type ABA levels. The results for these plant lines are representative of the other transgenic lines. At 2 days after imbibition, 79% of the DXS-suppressed plants have germinated compared with 10% for the DXS-overexpressing plants and 23% for the wild-type plants. At 5 days, 100% of the DXS-suppressed and wild-type plants have germinated, but only 46% of the DXS-overexpressing plants have germinated. In fact, throughout the 7 days that germination rates were followed, the DXS-overexpressing plants never reached 100% germination. Exposure to cold treatment prior to germination of S-17 and S-7 seeds substantially reduce such differences, suggesting that the effect observed is caused by the endogenous ABA levels in the seeds of those plants (data not shown). These results support the observation that changes in the endogenous ABA content of our transgenic plants correspond at least partially to an increment of a biologically active hormone that is reflected in one of the known physiological roles of ABA.

## DISCUSSION

Because isoprenoids are such ubiquitous and essential compounds, there have been intensive efforts to understand the pathways that lead to their production. All isoprenoids are derived from the precursor molecules IPP and DMAPP, which are produced in plants by either the cytoplasmic MVA or plastidic MEP pathways (19, 59). There has been substantial progress in the identification of the biosynthetic steps of the MEP pathway in the past few years. However, knowing the reaction steps involved in the MEP pathway is only the first step to fully understanding it. Fundamental aspects that need to be addressed are the regulatory and control points of the pathway. If DXS is a limiting enzyme of the MEP pathway, then altering its level will affect the quantity of IPP. Analogously, if the amount of plastidic IPP is limiting in the production of isoprenoids, alterations in the IPP level will have an effect on overall isoprenoid levels. One way to define experimentally the rate-limiting steps of a biosynthetic pathway is by using reverse genetics to make changes in specific sites of the pathway and then monitoring the corresponding changes in the end products (60). The experiments described in this article were designed to determine whether the first enzyme in the MEP pathway, DXS, is a limiting part of the production of plastidic IPP. Additionally, if DXS is limiting, we wanted to evaluate the effects of altering its level on seed germination, plant growth, and isoprenoid abundance.

The general finding for three divergent isoprenoid pathways is that by raising the level of DXS, the levels of isoprenoids are raised, and when the level of DXS is lowered, so are the levels of isoprenoids. Because the changes in DXS levels lead to changes in isoprenoid abundance, we propose that DXS is one of the limiting enzymes in the MEP pathway. It is likely that other co-limiting enzymes for each specific isoprenoid are present in the plant MEP pathway or further downstream, as we did not observe a linear relationship between changes in DXS and its end products. Additional limiting enzymes would also explain the differences observed in the relative increases among the isoprenoids monitored in this work. This is the case for chlorophyll and carotenoid content where increases in their levels are more restricted than others. To identify other limiting enzymes of the MEP pathway will require further analyses when the complete enzymatic steps are known and the relative effects of changing the levels of other MEP pathway enzymes have been evaluated. Recently, the MEP pathway has been intensively studied in bacteria where it was found that DXS also is as a limiting enzyme, and DXR is not (26, 34, 52). This conclusion is also supported by recent work on tomato fruit ripening. Lois *et al*. (25) found that DXS was limiting for carotenoid production over the previously identified limiting step of carotenoid biosynthesis (PSY1). They reasoned that DXS was limiting the amount of IPP available for carotenoid synthesis because even when there were increases in *PSY1* transcript levels, there were no increases in carotenoids without increases in DXS. The accumulated evidence indicates that DXS is one of the limiting steps in the MEP pathway of plants as well as in bacteria.

In this work we analyzed isoprenoids that are synthesized at very low levels such as hormones, and isoprenoids that are required in large quantities such as chlorophylls and carotenoids. In both cases a moderate change in the DXS level produced differences in the levels of the final isoprenoid products. Despite the fact that the biosynthetic pathway of GA has been intensively studied, there is no direct evidence that these hormones are actually synthesized via the MEP pathway. Our data suggest that GA biosynthesis depends, at least in part, on the IPP that comes from the MEP pathway. However, based on the expression of the *GA4* gene, the *cla1-1* mutant seems to contain active GA, which suggests that an additional source of IPP exists. Whether this IPP is the result of the import of cytosolic IPP (15) or if it comes from other sources remains to be established. Other groups have also looked at increasing or decreasing the levels of the hormones ABA (58, 61) and GA (62–64) by modulating the levels of enzymes in their individual post-IPP biosynthetic pathways. These studies have obtained relatively large changes in hormone levels with phenotypes complementing the proposed functions of these hormones, which demonstrate additional key regulatory steps in these biosynthetic pathways.

Much research has been devoted to increasing the levels of the isoprenoids $\alpha$-carotene, $\beta$-carotene (3, 65, 66), and $\alpha$-tocopherol (2) due, in part, to the importance of these molecules as precursors to vitamins needed by mammals. When total carotenoid content was increased (65, 66), there were concomitant

decreases in other isoprenoids. The results of these studies also support that the amount of IPP is limiting for isoprenoid production. In contrast, when only the relative quantity of isoprenoid end products was changed such as $\alpha$-carotene to $\beta$-carotene (3) or $\gamma$-tocopherol to $\alpha$-tocopherol (2), there were no other effects noted. By changing the amount of DXS and thus IPP levels, we are reporting the first instance in plants where a general increase or decrease in multiple plastidic isoprenoids was observed. Because moderate alterations in DXS levels (38–172%) lead to changes in all of the isoprenoids tested, it is apparent that the IPP from the MEP pathway is also limiting for the production of plastidic isoprenoids. The limiting role of IPP availability in isoprenoid production implies that to increase an individual isoprenoid without decreasing other isoprenoids requires a concurrent increase in IPP production.

Even though the levels of the different isoprenoids increase or decrease according to the level of DXS, the various isoprenoids do not change equally showing the complexity of the isoprenoid biosynthetic pathways that diverge from plastidic IPP. These results, together with the studies mentioned above wherein amounts of individual isoprenoids could be modulated through manipulation of genes in their post-IPP biosynthetic pathways, demonstrate that each of these post-IPP biosynthetic pathways has its own set of limiting and regulatory steps.

Aside from the measured changes in isoprenoid content, the DXS-overexpressing and -suppressed transgenic plants had close to normal phenotypes when grown in germination medium or in soil under optimal conditions. Significant differences were observed in the growth rates of the 8- and 26-day-old transgenic plants but not in the final plant size. These differences are not likely to be due solely to the observed changes in GA levels because changes of this hormone result in either dwarf or giant phenotypes (67). A possible explanation for the growth rate effects in the transgenic lines is that the plastidic IPP pathway receives its substrates directly from the Calvin cycle; therefore, changing the amount of IPP produced in these plants might have a direct effect on photosynthetic carbon availability to other pathways in the cell. Therefore, we hypothesized that the differences in growth rates are likely due to a pleiotropic effect of changes in carbon metabolism and hormone levels. On the other hand, the differences in germination rates can likely be directly correlated to the effects of either raised or lowered ABA levels. ABA plays a major role in setting and maintaining dormancy in seeds (56). The DXS-suppressed plants with less ABA content germinated rapidly, whereas the DXS-overexpressing plants had poor germination rates. These results are very similar to those obtained for ABA mutants of the post-IPP biosynthetic pathway (58, 61). In addition to the germination rates, transgenic plants containing 4-fold more ABA do not display any other phenotypes when grown under normal conditions. In contrast to what was reported in tomato, we could not observe overguttation or chlorosis in the leaves of these plants, when grown under normal conditions (58). It is possible that the levels of ABA in the transgenic lines are not sufficient to generate these phenotypes in *Arabidopsis* or that a specific growth condition could be required to display these phenotypes. We believe that a more detailed analysis of these plants could provide additional information about ABA regulation and function in plants. For example, the proposed regulation between biosynthesis and catabolism of ABA seems insufficient to prevent the ABA increments observed in the overexpressing plants. These results are similar to what was found with the ectopic expression of the tomato 9-*cis*-epoxycarotenoid dioxygenase enzyme, involved in the ABA biosynthetic pathway (58). Altering DXS levels, and hence IPP, manifested itself phenotypically in changes in growth rates, but not the final plant size, as well as having an effect on germination rates.

Varying the quantity of DXS may also lead to differences in the amounts of thiamin and pyridoxol because the product of DXS, DXP, is a precursor to these molecules as well as IPP. However, it is unlikely that either thiamin or pyridoxol deficiency gives rise to the observed phenotypes of these transgenic plants because the measurements of isoprenoid content and seedling size were performed in the presence of media containing vitamin supplements. As suggested in the initial studies of the *cla1-1* mutant (27, 28), alterations in the quantity of vitamins cannot account for the phenotypes that were observed in the transgenic plants.

Finally, since many isoprenoids are useful in medical, nutritional, or industrial applications and our ability to manipulate successfully metabolic pathways in plants continues to improve, isoprenoids are becoming a prime target for the production of commercially viable transgenic plants. By having isolated and characterized DXS (27, 28), the first gene in the MEP pathway of plants, we wanted to know if DXS was one of the limiting enzymes of the MEP pathway as it is in bacteria (24, 26, 34). We tested this idea by manipulating DXS levels in transgenic plants. We observed changes in the levels of a wide variety of isoprenoids, and these increases or decreases in isoprenoid levels followed the levels of DXS. From these results we conclude that DXS catalyzes one of the limiting steps of the MEP pathway. Also, since changes in DXS levels exert their effect on isoprenoid levels through changing the levels of IPP, it would appear that plastidic isoprenoid production is limited by the availability of IPP. Although other studies have shown increases or decreases in single isoprenoids (2, 3, 58, 61–64) or increases in one isoprenoid with decreases in others (65, 66), this is the first report that shows changes in several plastidic isoprenoid levels by altering the levels of the first enzyme in the MEP pathway, DXS.

*Acknowledgments*—We thank Analilia Arroyo Becerra, Dr. Yuji Kamiya, and Dr. Kenneth Luhersen for their critical comments on the manuscript and Dr. Brigitte Nowakowsky for expert technical help. BASFAG contributed to the characterization of transgenic plants without any participation in their generation.

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