

Up-Regulation of 1-Deoxy-D-Xylulose-5-Phosphate Synthase Enhances Production of Essential Oils in Transgenic Spike Lavender¹

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Spike lavender (*Lavandula latifolia*) is an aromatic shrub cultivated worldwide for the production of essential oils. The major constituents of these oils are monoterpenes, which are obtained from isopentenyl diphosphate and dimethylallyl diphosphate precursors through the plastidial methylerythritol phosphate (MEP) pathway and/or the cytosolic mevalonate pathway. 1-Deoxy-D-xylulose-5-P synthase (DXS) catalyzes the first step of the MEP pathway. A cDNA coding for the *Arabidopsis thaliana* DXS was constitutively expressed in spike lavender. Gas chromatography/mass spectrometry analyses revealed that transgenic plants accumulated significantly more essential oils compared to controls (from 101.5% to 359.0% and from 12.2% to 74.1% yield increase compared to controls in leaves and flowers, respectively). T₀ transgenic plants were grown for 2 years, self-pollinated, and the T₁ seeds obtained. The inheritance of the DXS transgene was studied in the T₁ generation. The increased essential oil phenotype observed in the transgenic T₀ plants was maintained in the progeny that inherited the DXS transgene. Total chlorophyll and carotenoid content in DXS progenies that inherited the transgene depended on the analyzed plant, showing either no variation or a significant decrease in respect to their counterparts without the transgene. Transgenic plants had a visual phenotype similar to untransformed plants (controls) in terms of morphology, growth habit, flowering, and seed germination. Our results demonstrate that the MEP pathway contributes to essential oil production in spike lavender. They also demonstrate that the DXS enzyme plays a crucial role in monoterpene precursor biosynthesis and, thus, in essential oil production in spike lavender. In addition, our results provide a strategy to increase the essential oil production in spike lavender by metabolic engineering of the MEP pathway without apparent detrimental effects on plant development and fitness.

Plant secondary metabolism is a source of both biologically and economically important compounds. Thus, constituents of essential oils are thought to be involved in plant-insect, plant-pathogen, and plant-plant interactions (Pichersky and Gershenzon, 2002). In addition, essential oils have an economic value as flavors, fragrances, and medicines (Verlet, 1993). Spike lavender (*Lavandula latifolia* Medicus), of the family Lamiaceae, is an aromatic shrub native to the Mediterranean region that is cultivated worldwide for the production of spike lavender oil. This oil is traditionally believed to be antibacterial, antifungal, carminative (smooth muscle relaxing), sedative, antidepressive, and effective for burns and insect bites (Font-Quer, 1978). Although the biological activities of this essen-

tial oil are still inconclusive, there are clinical data that support their traditional uses (Cavanagh and Wilkinson, 2002). The composition of spike lavender oil is determined mainly by the genetic make-up of each cultivar, although monoterpenes are always the major fraction, with three of them (linalool, cineol, and camphor) accounting for more than 80% of the total sample (Harborne and Williams, 2002). As in other Lamiaceae, these compounds seem to be synthesized and accumulated in the peltate glandular trichomes found on the aerial parts of the species (Hallahan, 2000).

Monoterpene biosynthesis can be divided into four phases (Mahmoud and Croteau, 2002; Dudareva et al., 2004): (1) construction of the basic C5 units isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP); (2) condensation of IPP and DMAPP by prenyltransferases to form geranyl diphosphate (GPP; C10); (3) conversion of GPP to the parent structure of the various monoterpene subfamilies, catalyzed by terpene synthases; and (4) transformation of the parent structures to various derivatives. The formation of IPP and DMAPP proceeds via two alternative pathways (Fig. 1): the classic cytosolic mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway (Rodriguez-Concepción and Boronat, 2002). The MEP pathway, localized in the plastids, is thought to provide IPP and DMAPP for monoterpene and sesquiterpene biosynthesis (Mahmoud and Croteau,

¹ This work was supported by Dirección General de Investigación Científica y Técnica, Madrid (project AGL2002-00977); by Generalitat Valenciana, Valencia, Spain (projects GV2001-020 and Grupos 03/102); and by a Formación de Profesorado Universitario Research Fellowship from the Spanish Ministerio de Educación y Cultura (to J.M.-B.).

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www.plantphysiol.org/cgi/doi/10.1104/pp.106.086355

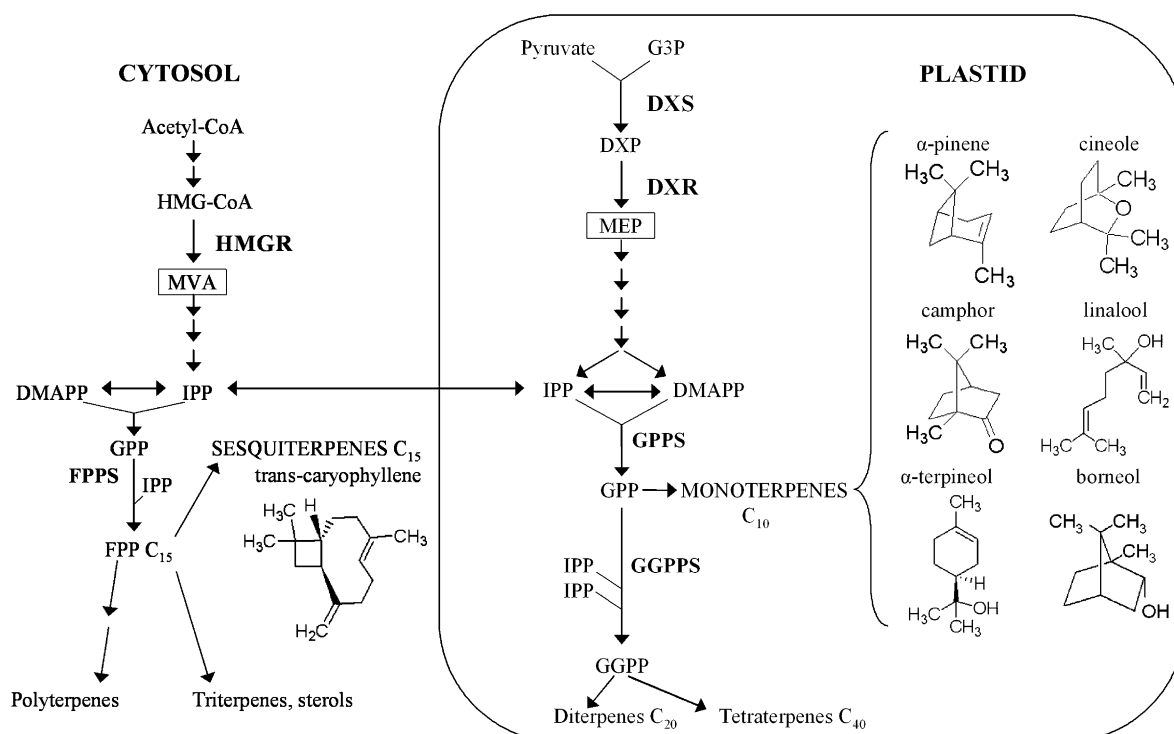


Figure 1. Isoprenoid biosynthesis in plants. Enzymes are indicated in boldface: FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; GPPS, GPP synthase; and HMGR, 3-hydroxy-3-methylglutaryl CoA reductase. The first intermediate specific to each pathway is boxed: FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; G3P, D-glyceraldehyde 3-P; and HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA. Chemical structures of representative mono- and sesquiterpenes analyzed are shown.

2002; Dudareva et al., 2005). Other studies, however, demonstrate that the MVA pathway can be the primary source of precursors for these compounds (Hampel et al., 2006). Cross-talk between the two pathways has also been documented (Schuhr et al., 2003; Hampel et al., 2005). Because of this, the relative contributions of each pathway to the biosynthesis of the various classes of terpenes remain uncertain.

Although studies on metabolic engineering of monoterpenes in Lamiaceae are scarce, investigations in peppermint (*Mentha x piperita*) suggest that the MEP pathway may be the main source of precursors (IPP and DMAPP) for monoterpenes in this family (Wildung and Croteau, 2005). Also, there are clear indications that precursor supply is a limiting factor in the biosynthesis of monoterpenes from this pathway (Mahmoud and Croteau, 2002). The MEP pathway (Rohmer, 2003) starts by the transketolase-type condensation of two carbons from pyruvate with glyceraldehyde-3-P to form 1-deoxy-D-xylulose-5-P (DXP), catalyzed by DXP synthase (DXS). The subsequent synthesis of MEP, catalyzed by DXP reductoisomerase (DXR), is the previous step for the formation of the C₅ units, following reactions in which the last enzyme of the MEP pathway (hydroxymehtylbutenyl diphosphate reductase) synthesizes IPP and DMAPP simultaneously (Fig. 1).

DXS has been postulated to catalyze a crucial step in the formation of plastid-derived isoprenoids (Lois et al., 2000; Estévez et al., 2001). In nonaromatic plant species, such as *Arabidopsis* (*Arabidopsis thaliana*) and tomato (*Lycopersicon esculentum*), the overexpression of DXS, which catalyzes the first step of the MEP pathway, resulted in elevated levels of carotenoids and other terpenoids (Estévez et al., 2001; Enfissi et al., 2005). On the other hand, up-regulation of DXR, another enzyme of MEP pathway, in transgenic peppermint plants resulted in a 50% increase in essential oil yield (Mahmoud and Croteau, 2001). Whether or not the concentration of certain isoprenoid precursors is limiting for the production of monoterpenes probably depends on the species, the tissue, and the physiological state of the plant (Aharoni et al., 2005).

To investigate the effect of an increase of monoterpene precursors on the final production of essential oils, we targeted the first step in the MEP pathway by overexpressing the DXS cDNA from *Arabidopsis* in spike lavender. This approach also allowed us to study the contribution of the MEP pathway in the biosynthesis of essential oils in aromatic plants.

To date, selection of high essential oil-yielding spike lavender has been exclusively based on conventional breeding programs. Progress in biotechnology offers an alternative and promising approach to improve

spike lavender oil production by genetic engineering. Successful application of this approach will depend on the transgene being expressed and inherited in a stable and predictable way. Because of this, we also studied the expression and inheritance of the *DXS* gene in T_1 progeny from transgenic lines that flowered 2 years after growing at the greenhouse.

RESULTS

Generation and Evaluation of Transgenic Spike Lavender

Using an *Agrobacterium*-based leaf culture protocol set up in our laboratory (Nebauer et al., 2000), spike lavender transgenic plants expressing the *DXS* gene from *Arabidopsis* were generated. The *DXS* gene was under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter. Resistance to kanamycin was used to select putative transformants.

All kanamycin-resistant plants were first screened by PCR for the presence of neomycin phosphotransferase II (*nptII*) and *DXS* genes (data not shown). All *nptII*⁺/*DXS*⁺ plants were cloned, acclimatized to *ex vitro* conditions, and transferred to the greenhouse for further analyses. Seven independent primary transformants (T_0), designed as DXS1 to DXS7, were obtained. The transgenic plants did not show any obvious phenotypic differences compared to untrans-

formed greenhouse-grown plants (Fig. 2, A and B) in terms of morphology, growth habit, flowering, and seed germination.

The number of transgene inserts was determined by Southern blotting using both *nptII* and *DXS* probes. Five out of the seven independent transgenic lines (DXS1, DXS2, DXS3, DXS5, and DXS7) presented single-copy insertions, while transgenic lines DXS4 and DXS6 had two copies of the *DXS* gene (Fig. 3A). Identical hybridization patterns were obtained for the *nptII* transgene (data not shown), corroborating the insertion of the complete T-DNA.

To correlate phenotypes of the *DXS* lines with gene expression, mRNA and protein levels of *DXS* were determined for transgenic T_0 plants. Total RNA was isolated from leaves and flowers, and expression of the transgenic *DXS* mRNA was determined by northern blot analyses. Figure 3, B and C, shows that there are large differences in expression levels of the *DXS* cDNA among the independent transgenic lines. In leaves (Fig. 3B), all lines but one (DXS3) showed detectable levels of *DXS* mRNA, with lines 1, 4, 5, 6, and 7 showing the highest levels. Again, transgene expression in flowers (Fig. 3C) was line dependent, with lines DXS1, 4, 6, and 7 showing the highest levels. Under the conditions used in our experiments, the *DXS* probe did not cross-react with the spike lavender *DXS* transcript because there was no detectable band in the

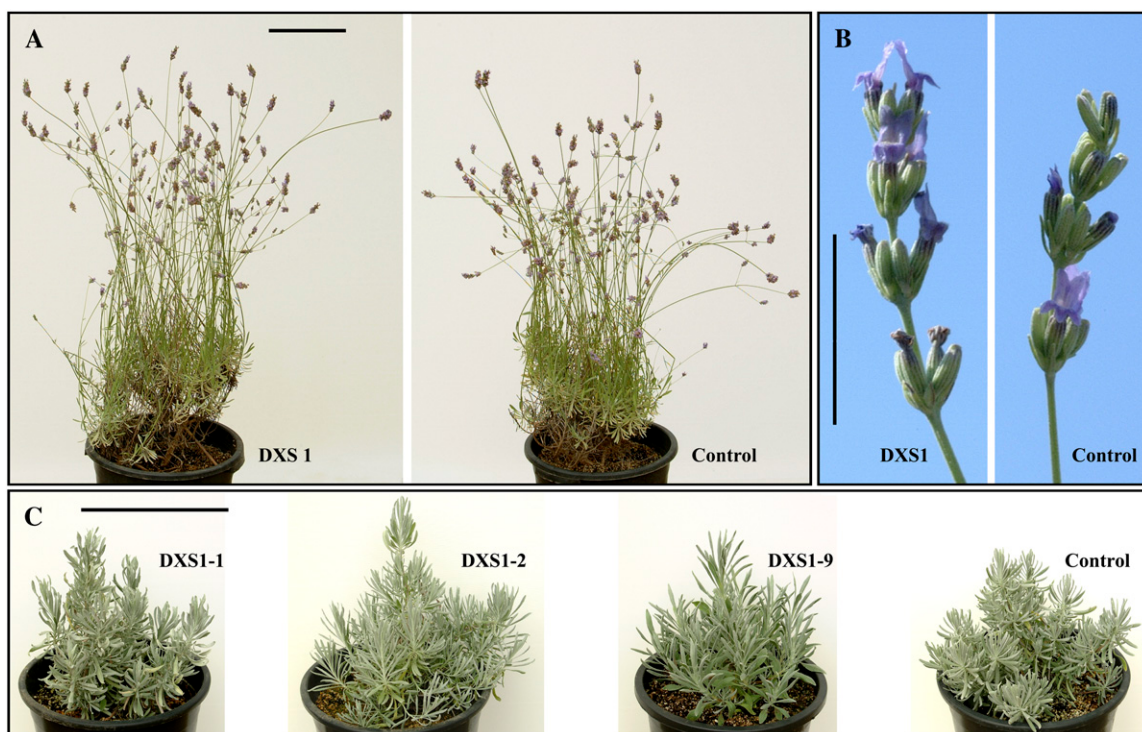


Figure 2. Spike lavender plants grown in the greenhouse. A, Gross morphology at the flowering stage of 4-year-old transgenic T_0 DXS1 and control plants; bar = 20 cm. B, Flower morphology of transgenic T_0 DXS1 and control plants; bar = 2.5 cm. C, Gross morphology of 8-month-old DXS1 progenies that inherited (DXS1-1 and DXS1-2) or not (DXS1-9) the *DXS* gene and a control plant; bar = 20 cm.

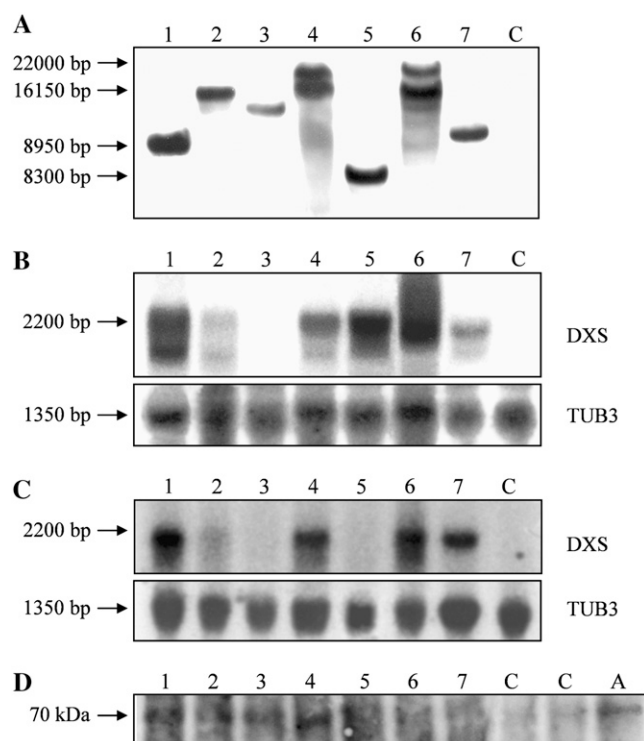


Figure 3. Molecular analyses of transgenic T_0 spike lavender plants. A, Southern-blot hybridization of *DXS* gene. B and C, Expression of *DXS* in leaves (B) and flowers (C) of transgenic lines; the expression of the *TUB3* gene is shown to verify equal loading. D, Western-blot analysis of the Arabidopsis *DXS* protein in spike lavender and Arabidopsis. Lane 1, DXS1; lane 2, DXS2; lane 3, DXS3; lane 4, DXS4; lane 5, DXS5; lane 6, DXS6; lane 7, DXS7; lane C, control; and lane A, Arabidopsis.

control lane (Fig. 3B). Western blotting performed on extracts obtained from young leaves demonstrated that the processed *DXS* protein was detected in control and transgenic lines, but the latter showed a moderately higher level (Fig. 3D).

Overexpression of the *DXS* Gene Increases Essential Oil Production in T_0 Spike Lavender Plants

Essential oil analyses were accomplished by hydro-distillation of air-dried leaves or flowers followed by gas chromatography (GC) separation of components and quantification using internal standards. Identification of the oil components was corroborated by GC/mass spectrometry (MS). The most common components usually found in spike lavender oils were present in the oil samples analyzed (Harborne and Williams, 2002). Twenty-eight constituents were identified, accounting for 81% to 93% of the total oils. In both transgenic and control plants, the essential oils consisted mainly of monoterpenes (99.4%–99.9% and 95.6%–99.5% in leaves and flowers, respectively). The most abundant fractions were oxygenated and hydrocarbon monoterpenes. The oxygenated monoterpenes ranged from 96.4% to 98.0% in leaves and from 92.8% to 98.3% in flowers. The hydrocarbon monoterpenes were less abundant, ranging from 2.0% to 3.6% in leaves and from 1.7% to 7.2% in flowers. The sesquiterpene fraction was minimal in leaves (0.002%–0.01%), whereas in flowers the concentration ranged from 0.1% to 4.2%.

Essential oil yield (milligram per gram dried tissue) in leaves and flowers of spike lavender was markedly increased in most of the transgenic plants that expressed the *DXS* cDNA (Table I). In leaves, six out of seven transgenic lines accumulated significantly more essential oils than controls (from 101.5% to 359.0% yield increase in DXS7 and DXS1, respectively). In both transgenic and control plants, essential oil production in flowers was higher than in leaves. Moreover, flowers from all transgenic plants produced significantly more essential oils than the controls, but the yield increase (from 17.2% to 74.1% in DXS2 and DXS1, respectively) was lower than in leaves (Table I). Both hydrocarbon and oxygenated monoterpenes contributed to the increased oil yield of transgenic plants, but the latter showed the highest increases in relation

Table I. Essential oil yield and monoterpene production (milligram per gram dried weight) in leaves (L) and flowers (F) of control and transgenic T_0 spike lavender plants transformed with the Arabidopsis *DXS* gene

Reported values for transgenic plants represent the means $\pm$ SD of four measurements. The control value represents the average of four wild-type plants with four measurements each. Within each column, values followed by different letters are significantly different according to Tukey's test at $P \leq 0.05$.

Plants	Oil Yield		Monoterpenes			
			Hydrocarbons		Oxygenated	
	L	F	L	F	L	F
Control	12.72 $\pm$ 6.83 a	35.88 $\pm$ 2.07 a	1.44 $\pm$ 0.40 a	5.62 $\pm$ 0.38 b	11.18 $\pm$ 6.44 a	29.12 $\pm$ 1.72 a
DXS1	58.39 $\pm$ 0.84 e	62.47 $\pm$ 0.33 e	2.92 $\pm$ 0.09 d	6.14 $\pm$ 0.30 c	55.22 $\pm$ 0.87 e	55.47 $\pm$ 0.02 f
DXS2	34.98 $\pm$ 0.93 c	42.04 $\pm$ 0.04 b	2.15 $\pm$ 0.02 b	8.29 $\pm$ 0.11 e	32.61 $\pm$ 0.92 cd	32.33 $\pm$ 0.03 b
DXS3	14.42 $\pm$ 0.75 a	46.30 $\pm$ 0.28 c	2.05 $\pm$ 0.30 b	7.80 $\pm$ 0.35 d	12.17 $\pm$ 1.11 a	37.05 $\pm$ 0.03 c
DXS4	25.96 $\pm$ 0.45 b	51.07 $\pm$ 0.52 d	2.33 $\pm$ 0.04 bc	8.57 $\pm$ 0.11 e	23.54 $\pm$ 0.45 b	39.35 $\pm$ 0.54 d
DXS5	41.46 $\pm$ 1.27 d	45.08 $\pm$ 0.32 c	2.81 $\pm$ 0.11 d	4.98 $\pm$ 0.22 a	38.35 $\pm$ 1.17 d	38.85 $\pm$ 0.20 d
DXS6	29.31 $\pm$ 1.41 bc	62.08 $\pm$ 0.11 e	2.55 $\pm$ 0.02 cd	7.44 $\pm$ 0.05 d	26.67 $\pm$ 1.41 bc	51.96 $\pm$ 0.15 e
DXS7	25.63 $\pm$ 0.51 b	43.06 $\pm$ 0.09 b	2.08 $\pm$ 0.04 b	5.99 $\pm$ 0.01 c	23.37 $\pm$ 0.53 b	35.81 $\pm$ 0.10 c

Table II. Percentage of major essential oil constituents in leaves (L) and flowers (F) of control and transgenic *T₀* spike lavender plants transformed with the *Arabidopsis* DXS gene

Reported percentages for transgenic plants represent the means $\pm$ SD of four measurements. The control value represents the average of four wild-type plants with four measurements each.

Plants	α - and β -Pinenes		Cineole		Camphor	
	L	F	L	F	L	F
Control	1.58 $\pm$ 0.68	2.01 $\pm$ 1.12	39.66 $\pm$ 11.28	16.41 $\pm$ 3.75	43.77 $\pm$ 11.09	25.89 $\pm$ 7.56
DXS1	1.73 $\pm$ 0.28	1.32 $\pm$ 0.54	46.25 $\pm$ 1.14	16.70 $\pm$ 0.23	39.12 $\pm$ 1.25	19.03 $\pm$ 0.23
DXS2	1.70 $\pm$ 0.11	2.88 $\pm$ 0.37	59.43 $\pm$ 3.17	34.13 $\pm$ 0.07	23.75 $\pm$ 1.08	18.19 $\pm$ 0.09
DXS3	1.61 $\pm$ 0.20	2.88 $\pm$ 1.15	49.86 $\pm$ 1.24	34.33 $\pm$ 0.63	34.07 $\pm$ 1.73	17.01 $\pm$ 0.19
DXS4	1.82 $\pm$ 0.26	1.29 $\pm$ 0.01	37.43 $\pm$ 0.29	20.18 $\pm$ 0.4	47.47 $\pm$ 0.50	28.32 $\pm$ 0.03
DXS5	2.08 $\pm$ 0.05	0.54 $\pm$ 0.08	42.01 $\pm$ 0.21	8.56 $\pm$ 0.15	41.91 $\pm$.38	20.45 $\pm$ 0.04
DXS6	3.49 $\pm$ 0.16	0.74 $\pm$ 0.00	41.18 $\pm$ 0.37	11.14 $\pm$ 0.03	42.17 $\pm$ 0.12	29.31 $\pm$ 0.00
DXS7	1.99 $\pm$ 0.09	0.49 $\pm$ 0.02	54.26 $\pm$ 0.32	17.68 $\pm$ 0.09	29.77 $\pm$ 0.26	16.67 $\pm$ 0.02

Plants	Linalool		α -Terpineol		Borneol	
	L	F	L	F	L	F
Control	0.02 $\pm$ 0.00	29.80 $\pm$ 10.21	0.98 $\pm$ 0.28	0.87 $\pm$ 0.94	2.09 $\pm$ 0.60	5.11 $\pm$ 2.12
DXS1	0.02 $\pm$ 0.00	45.92 $\pm$ 0.25	1.10 $\pm$ 0.12	0.34 $\pm$ 0.01	2.29 $\pm$ 0.37	1.94 $\pm$ 0.00
DXS2	0.02 $\pm$ 0.00	12.19 $\pm$ 0.01	1.54 $\pm$ 0.07	0.94 $\pm$ 0.02	2.75 $\pm$ 0.12	5.81 $\pm$ 0.00
DXS3	0.02 $\pm$ 0.00	15.95 $\pm$ 0.22	0.81 $\pm$ 0.09	0.90 $\pm$ 0.01	2.53 $\pm$ 0.28	5.19 $\pm$ 0.11
DXS4	0.01 $\pm$ 0.00	29.10 $\pm$ 0.14	0.81 $\pm$ 0.05	1.05 $\pm$ 0.02	1.70 $\pm$ 0.11	3.22 $\pm$ 0.01
DXS5	0.02 $\pm$ 0.00	53.04 $\pm$ 0.23	1.10 $\pm$ 0.03	0.34 $\pm$ 0.03	2.19 $\pm$ 0.06	1.80 $\pm$ 0.03
DXS6	0.02 $\pm$ 0.00	43.29 $\pm$ 0.02	0.65 $\pm$ 0.02	0.54 $\pm$ 0.02	2.05 $\pm$ 0.07	2.57 $\pm$ 0.03
DXS7	0.02 $\pm$ 0.00	48.16 $\pm$ 0.03	0.52 $\pm$ 0.02	0.43 $\pm$ 0.07	1.44 $\pm$ 0.05	1.37 $\pm$ 0.06

to the controls (increase averages of 67.6% versus 171% and 25.1% versus 42.7% in leaves and flowers, respectively). Table II summarizes the percentages of major essential oil constituents in transgenic and control spike lavender plants. In transgenic lines, these percentages were within the range already described in spike lavender essential oil and similar to the controls (Table II). Note that linalool, a major constituent in spike lavender oils from flowers, is a minor constituent in the leaf oils ($\leq 0.02\%$). Thus, a consistent increase of a specific essential oil constituent was not observed in leaves of transgenic plants as compared to controls.

Inheritance of the DXS Gene in *T₁* Spike Lavender Plants Correlated with a Higher Essential Oil Yield

To characterize integration of the transforming cDNA into the spike lavender genome and its inheritance, we analyzed *T₁* plants derived from self-pollinated seeds of the *T₀* lines that flowered after 2 years growing in the greenhouse. Inheritance of the *DXS* and *nptII* transgenes was determined by PCR (Table III). Transgenic lines DXS1, 2, and 5, having only one *DXS* copy according to the Southern blot, showed the 3:1 segregation, whereas DXS6, having two copies, showed a 15:1 segregation (Fig. 3A). However, line DXS4, which according to the Southern blotting also had two copies (Fig. 3A), showed a 3:1 segregation, suggesting that in this plant both copies were integrated in the same chromosome. Segregation of the *nptII* gene was concordant with the *DXS* gene (data not shown). Thus, both transgenes behave as typical dominant, linked genes. *T₁* progenies from the highest essential oil-

producing lines (DXS1, 4, and 6) were selected for further molecular and phenotypic analyses.

Southern blotting patterns of the DXS1-*T₁* plants that inherited the transgene were similar to that of the single-copy parent (Fig. 4A). As expected, progeny of the two copy parent lines (DXS4 and 6) that inherited the transgene showed one or two copies in the corresponding Southern blot (Fig. 4, C and E). Note, however, that most of the DXS4-*T₁* positive plants inherited the two copies of the *DXS* transgene, corroborating the 3:1 segregation obtained from PCR analyses (Table III). Data from the Southern analysis is consistent with the northern analysis: plant material exhibiting transgene expression also exhibited the complete DNA repertoire, whereas material from nontransgene-expressing plants contained no detectable signal (Fig. 4).

Table III. Segregation of *DXS* gene in *T₁* plants from self-pollinated transgenic *T₀* lines of spike lavender plants

DXS⁺ and DXS⁻ = PCR positive and PCR negative, respectively. A chi-squared value >3.84 indicates a significant deviation from the expected ratio ($P = 0.05$).

Transgenic <i>T₀</i> Lines	No. of Plants		Chi-Squared Value for Each Ratio	
	DXS ⁺	DXS ⁻	3:1 (One Insert)	15:1 (Two Inserts)
DXS1	58	24	0.58	70.27
DXS2	18	6	0.50	25.60
DXS4	55	25	1.35	81.12
DXS5	16	3	0.44	1.55
DXS6	38	3	5.93	0.00

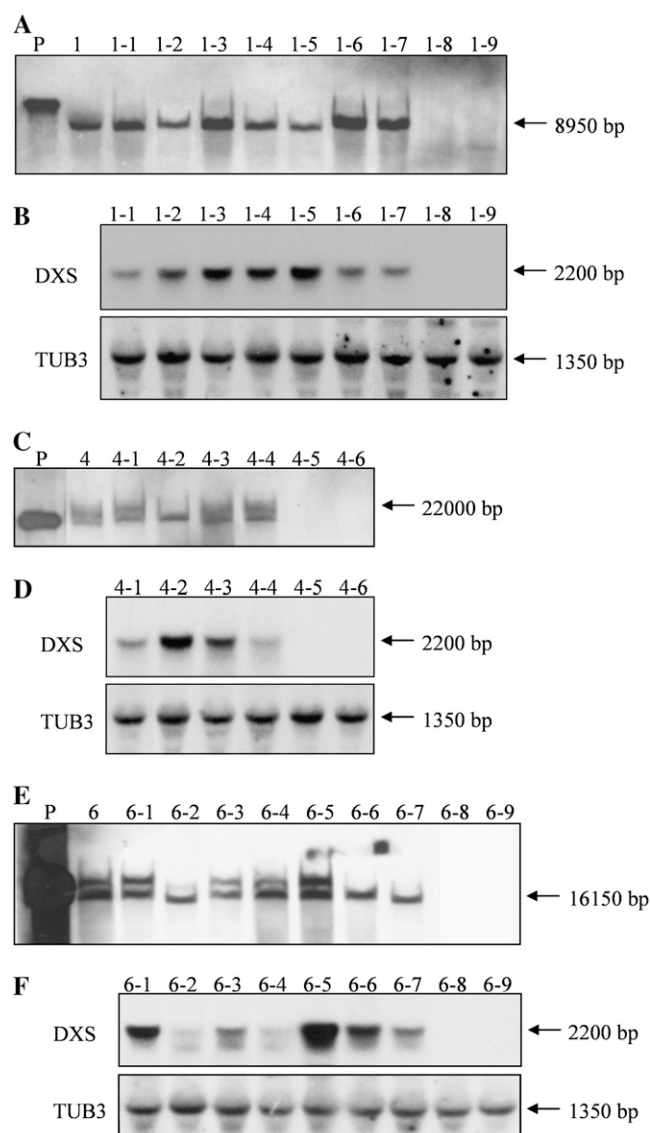


Figure 4. Molecular analyses of transgenic T_1 spike lavender plants. A, C, and E, Southern-blot hybridization of *DXS* gene. B, D, and F, Northern blotting of *DXS* gene; the expression of the *TUB3* gene is shown to verify equal loading. Lane P, plasmid; lane 1, parental T_0 DXS1; lanes 1-1 to 1-9, selected progenies of DXS1 plant; lane 4, parental T_0 DXS4; lanes 4-1 to 4-6, selected progenies of DXS4 plant; lane 6, parental T_0 DXS6; lanes 6-1 to 6-9, selected progenies of DXS6 plant.

Essential oil analyses of T_1 plants showed that the enhanced monoterpene content observed in the first generation was stable in the subsequent generation (Tables I and IV). Regardless of the T_0 parental line, most of the T_1 plants that inherited the *DXS* gene maintained their elevated essential oil phenotype; average oil yield increases in DXS1, DXS4, and DXS6 progenies that inherited the transgene were 77.3%, 38.4%, and 48.8% with respect to their T_1 counterparts without the transgene (Table IV). Corroborating the results obtained in transgenic T_0 plants, oxygenated

monoterpenes constituted the main contribution to the increased oil yield (Tables I and IV). Note that northern-blot analyses indicated that increases in the expression of the *DXS* cDNA from *Arabidopsis* (Fig. 4) roughly paralleled increases in the monoterpene content of progeny that inherited the *DXS* gene (Table IV). The essential oil compositions of the T_1 samples were similar to the T_0 counterparts (Tables II and V). All these results demonstrate that *DXS* transgene was stably transmitted to the progeny.

Overexpression of the *DXS* Gene Does Not Increase Chlorophyll and Carotenoid Content of the Spike Lavender Leaves

Experimental evidence has clearly demonstrated that plastid isoprenoids involved in photosynthesis, such as carotenoids and the phytol moiety of chlorophylls, are derived from the MEP pathway (Lichtenthaler, 1999). Thus, we quantified these compounds in spike lavender leaves from T_1 progenies of DXS1, 4, and 6 lines that did or did not inherit the *DXS* gene. Total chlorophyll and carotenoid content in DXS progenies that inherited the transgene depended on the analyzed

Table IV. Essential oil yield and monoterpene production (milligram per gram dried weight) from leaves of representative transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic DXS1, DXS4, and DXS6 lines

Reported values for each T_1 plant represent the mean $\pm$ SD of four measurements. Within each column and progeny (DXS1, DXS4, and DXS6), values followed by different letters are significantly different according to Tukey's test at $P \leq 0.05$. *, T_1 plants that did not inherit the *DXS* transgene.

Plants	Oil Yield	Monoterpenes	
		Hydrocarbons	Oxygenated
DXS1-1	53.97 $\pm$ 1.57 f	5.29 $\pm$ 0.06 e	48.21 $\pm$ 1.60 e
DXS1-2	45.22 $\pm$ 3.04 d	5.22 $\pm$ 0.09 c	39.80 $\pm$ 2.95 c
DXS1-3	57.54 $\pm$ 0.05 g	6.03 $\pm$ 0.11 c	51.06 $\pm$ 0.16 f
DXS1-4	59.31 $\pm$ 0.50 g	5.74 $\pm$ 0.12 d	53.20 $\pm$ 0.53 f
DXS1-5	44.10 $\pm$ 2.60 cd	6.00 $\pm$ 0.06 e	37.75 $\pm$ 2.54 c
DXS1-6	48.59 $\pm$ 0.08 e	5.77 $\pm$ 0.06 d	42.59 $\pm$ 0.08 d
DXS1-7	41.87 $\pm$ 1.70 c	3.72 $\pm$ 0.12 a	37.68 $\pm$ 1.56 c
DXS1-8*	31.93 $\pm$ 2.35 b	4.22 $\pm$ 0.18 b	26.68 $\pm$ 2.12 b
DXS1-9*	24.56 $\pm$ 0.80 a	4.30 $\pm$ 0.17 b	20.05 $\pm$ 0.63 a
DXS4-1	17.45 $\pm$ 1.20 c	3.48 $\pm$ 0.23 cd	13.73 $\pm$ 0.95 c
DXS4-2	18.76 $\pm$ 1.18 c	3.77 $\pm$ 0.09 d	14.77 $\pm$ 1.16 cd
DXS4-3	18.69 $\pm$ 1.50 c	3.27 $\pm$ 0.13 bc	15.18 $\pm$ 1.34 d
DXS4-4	10.84 $\pm$ 0.19 a	2.63 $\pm$ 0.37 a	8.15 $\pm$ 0.18 a
DXS4-5*	12.98 $\pm$ 0.90 b	3.11 $\pm$ 0.03 b	9.80 $\pm$ 0.88 b
DXS4-6*	10.77 $\pm$ 0.53 a	2.64 $\pm$ 0.37 a	8.05 $\pm$ 0.15 a
DXS6-1	30.64 $\pm$ 0.18 f	4.16 $\pm$ 0.08 f	26.20 $\pm$ 0.10 f
DXS6-2	18.01 $\pm$ 1.03 c	2.64 $\pm$ 0.16 a	15.11 $\pm$ 0.85 c
DXS6-3	22.38 $\pm$ 0.77 d	3.37 $\pm$ 0.14 d	18.81 $\pm$ 0.64 d
DXS6-4	22.65 $\pm$ 0.82 d	3.69 $\pm$ 0.15 e	18.84 $\pm$ 0.66 d
DXS6-5	29.22 $\pm$ 0.04 e	4.59 $\pm$ 0.06 h	24.51 $\pm$ 0.07 e
DXS6-6	39.95 $\pm$ 1.22 g	4.38 $\pm$ 0.19 g	35.07 $\pm$ 0.99 g
DXS6-7	15.50 $\pm$ 0.04 a	3.10 $\pm$ 0.04 c	12.14 $\pm$ 0.07 a
DXS6-8*	16.63 $\pm$ 0.24 b	2.92 $\pm$ 0.02 b	13.52 $\pm$ 0.23 b
DXS6-9*	17.61 $\pm$ 0.28 c	2.53 $\pm$ 0.07 a	14.91 $\pm$ 0.34 c

Table V. Percentage of major essential oil constituents from leaves of representative transgenic *T₁* spike lavender plants obtained from controlled self-pollination of *T₀* transgenic *DXS1*, *DXS4*, and *DXS6* linesReported percentages represent the means $\pm$ SD of four measurements. *, *T₁* plants that did not inherit the *DXS* transgene.

Plants	α - and β -Pinenes	Cineole	Camphor	Linalool	α -Terpineol	Borneol	Total
DXS1-1	8.25 $\pm$ 0.13	46.50 $\pm$ 0.20	26.07 $\pm$ 0.13	0.07 $\pm$ 0.00	2.73 $\pm$ 0.04	1.65 $\pm$ 0.07	94.79 $\pm$ 0.13
DXS1-2	4.24 $\pm$ 0.09	49.40 $\pm$ 0.31	29.06 $\pm$ 0.25	0.06 $\pm$ 0.00	1.71 $\pm$ 0.06	2.37 $\pm$ 0.04	94.72 $\pm$ 0.10
DXS1-3	6.25 $\pm$ 0.06	40.82 $\pm$ 0.08	33.05 $\pm$ 0.05	0.08 $\pm$ 0.00	2.02 $\pm$ 0.01	3.69 $\pm$ 0.00	95.22 $\pm$ 0.09
DXS1-4	7.15 $\pm$ 0.68	47.27 $\pm$ 2.11	26.66 $\pm$ 0.59	0.07 $\pm$ 0.00	2.59 $\pm$ 0.06	2.09 $\pm$ 0.08	94.96 $\pm$ 1.93
DXS1-5	4.04 $\pm$ 0.08	32.96 $\pm$ 0.97	40.76 $\pm$ 0.85	0.08 $\pm$ 0.00	1.26 $\pm$ 0.02	6.94 $\pm$ 0.17	94.35 $\pm$ 0.51
DXS1-6	5.53 $\pm$ 0.24	46.06 $\pm$ 0.10	29.36 $\pm$ 0.19	0.07 $\pm$ 0.00	1.81 $\pm$ 0.08	2.47 $\pm$ 0.02	94.01 $\pm$ 0.25
DXS1-7	5.33 $\pm$ 0.10	42.57 $\pm$ 0.23	29.76 $\pm$ 0.16	0.06 $\pm$ 0.01	1.81 $\pm$ 0.08	5.39 $\pm$ 0.19	93.59 $\pm$ 0.36
DXS1-8*	6.71 $\pm$ 0.24	29.76 $\pm$ 0.15	35.67 $\pm$ 0.29	0.10 $\pm$ 0.00	1.48 $\pm$ 0.06	5.78 $\pm$ 0.20	91.70 $\pm$ 0.36
DXS1-9*	6.79 $\pm$ 0.19	35.24 $\pm$ 0.50	38.21 $\pm$ 1.18	0.09 $\pm$ 0.00	1.88 $\pm$ 0.06	1.85 $\pm$ 0.08	94.30 $\pm$ 0.44
DXS4-1	7.46 $\pm$ 0.05	38.86 $\pm$ 0.32	30.45 $\pm$ 0.06	0.08 $\pm$ 0.00	2.32 $\pm$ 0.04	3.70 $\pm$ 0.18	94.26 $\pm$ 0.25
DXS4-2	8.23 $\pm$ 0.09	37.70 $\pm$ 0.51	30.10 $\pm$ 0.22	0.09 $\pm$ 0.00	2.11 $\pm$ 0.05	4.76 $\pm$ 0.03	93.84 $\pm$ 0.22
DXS4-3	6.99 $\pm$ 0.32	42.53 $\pm$ 1.2	27.60 $\pm$ 1.09	0.08 $\pm$ 0.00	2.54 $\pm$ 0.02	3.55 $\pm$ 0.22	93.71 $\pm$ 0.69
DXS4-4	4.03 $\pm$ 0.90	21.75 $\pm$ 0.72	53.63 $\pm$ 0.96	0.17 $\pm$ 0.00	0.97 $\pm$ 0.02	3.59 $\pm$ 0.16	92.62 $\pm$ 0.14
DXS4-5*	5.98 $\pm$ 0.08	25.53 $\pm$ 0.90	43.90 $\pm$ 0.51	0.20 $\pm$ 0.01	1.12 $\pm$ 0.03	5.27 $\pm$ 0.18	91.76 $\pm$ 0.59
DXS4-6*	4.06 $\pm$ 0.90	21.75 $\pm$ 0.72	53.63 $\pm$ 0.96	0.17 $\pm$ 0.00	0.97 $\pm$ 0.03	3.59 $\pm$ 0.16	92.64 $\pm$ 0.40
DXS6-1	7.59 $\pm$ 0.08	41.79 $\pm$ 0.20	31.17 $\pm$ 0.06	0.13 $\pm$ 0.01	1.59 $\pm$ 0.03	2.09 $\pm$ 0.02	93.63 $\pm$ 0.20
DXS6-2	6.14 $\pm$ 0.16	46.65 $\pm$ 0.13	27.81 $\pm$ 0.07	0.08 $\pm$ 0.00	1.82 $\pm$ 0.02	1.97 $\pm$ 0.06	93.84 $\pm$ 0.25
DXS6-3	7.87 $\pm$ 0.05	39.93 $\pm$ 1.07	31.79 $\pm$ 0.72	0.09 $\pm$ 0.00	1.67 $\pm$ 0.04	2.22 $\pm$ 0.05	93.19 $\pm$ 0.53
DXS6-4	6.97 $\pm$ 0.19	40.81 $\pm$ 0.53	32.01 $\pm$ 0.21	0.18 $\pm$ 0.03	1.67 $\pm$ 0.01	2.81 $\pm$ 0.02	93.38 $\pm$ 0.35
DXS6-5	9.27 $\pm$ 0.12	33.07 $\pm$ 0.11	35.87 $\pm$ 0.31	0.16 $\pm$ 0.00	1.47 $\pm$ 0.03	2.45 $\pm$ 0.03	92.28 $\pm$ 0.18
DXS6-6	7.14 $\pm$ 0.19	38.91 $\pm$ 0.11	33.35 $\pm$ 0.26	0.10 $\pm$ 0.01	1.97 $\pm$ 0.09	3.36 $\pm$ 0.06	94.53 $\pm$ 0.11
DXS6-7	8.74 $\pm$ 0.07	34.79 $\pm$ 0.73	33.75 $\pm$ 0.27	0.07 $\pm$ 0.00	2.06 $\pm$ 0.05	2.86 $\pm$ 0.14	94.18 $\pm$ 0.61
DXS6-8*	7.45 $\pm$ 0.16	37.42 $\pm$ 0.37	34.69 $\pm$ 0.12	0.12 $\pm$ 0.01	2.31 $\pm$ 0.04	2.39 $\pm$ 0.10	94.87 $\pm$ 0.10
DXS6-9*	8.36 $\pm$ 0.26	41.01 $\pm$ 0.94	29.47 $\pm$ 0.39	0.18 $\pm$ 0.02	1.50 $\pm$ 0.01	2.26 $\pm$ 0.07	92.89 $\pm$ 0.25

plant, showing no variation or a significant decrease respect to their counterparts without the transgene (Table VI). Irrespective of these variations in the photosynthetic pigments, none of the plants displayed visual phenotypes as compared to untransformed controls (Fig. 2C).

DISCUSSION

In higher plants, the common building C5 units for the synthesis of isoprenoid-derived primary and secondary metabolites are IPP and DMAPP. The two pathways leading to the biosynthesis of these C5 units are the cytosolic MVA and the plastidic MEP pathways (Rodríguez-Concepción and Boronat, 2002). Among the metabolites derived from the latter pathway are thought to be monoterpenes (Mahmoud and Croteau, 2002), the main chemical constituents of the essential oil from spike lavender.

Although DXR is the first committed step for terpenoid biosynthesis through the MEP pathway (Carretero-Paulet et al., 2002), several studies support that DXS, the first enzyme of this pathway, also has an important role for isoprenoid biosynthesis in several organisms, including bacteria and plants (Guevara-García et al., 2005, and refs. therein). Nevertheless, it has not yet been shown that this enzyme has a crucial function in the biosynthesis of monoterpenes in aromatic plants. Because of this, we have used the *Arabidopsis* *DXS* gene under the control of the constitutive CaMV 35S

promoter to test whether increased precursor availability could improve essential oil yield in spike lavender. We found that overexpressing DXS enhanced essential oil production in transgenic *T₀* spike lavender (from 100% to 359% and from 12.2% to 74.1% yield increase compared to controls in leaves and flowers, respectively), suggesting that this enzyme also plays a crucial role in monoterpene production. Our results also support the involvement of the MEP pathway in the biosynthesis of essential oils in aromatic plants, as previously suggested (Mahmoud and Croteau, 2002). This does not exclude that the MVA pathway could provide IPP precursors for monoterpene biosynthesis in these plants, as has been reported in strawberry fruits and foliage (Hampel et al., 2006).

Results reported here on DXS overexpression in spike lavender corroborate those obtained by up-regulation of another enzyme of the MEP pathway in peppermint (Mahmoud and Croteau, 2001). These authors showed that up-regulation of DXR, which catalyzes the second step of the MEP pathway, lead to a 50% increase of essential oils without substantial changes in monoterpene composition. These coincident results seem to be logical because the expression of the *DXS* gene closely parallels that of the *DXR* gene (Carretero-Paulet et al., 2002).

Despite the increased-monoterpene phenotype of the transgenic plants, essential oil profiles obtained from transgenic and control plants were quite similar to each other. It is well known that the variability in the spike lavender oil composition is primarily genotype

Table VI. Total chlorophyll and carotenoids content (milligram per gram fresh weight) in leaves of representative transgenic T_1 spike lavender plants, obtained from controlled self-pollination of T_0 transgenic *DXS1*, *DXS4*, and *DXS6* lines

Reported values for each T_1 plant represent the mean $\pm$ SD of at least three measurements. Within each column and progeny (*DXS1*, *DXS4*, and *DXS6*), values followed by different letters are significantly different according to Tukey's test at $P \leq 0.05$. *, T_1 plants that did not inherit the *DXS* transgene.

Plants	Chlorophylls (a + b)	Carotenoids
<i>DXS1</i> -1	2.13 $\pm$ 0.15 a	0.40 $\pm$ 0.03 abc
<i>DXS1</i> -2	1.84 $\pm$ 0.05 cd	0.35 $\pm$ 0.04 cd
<i>DXS1</i> -3	1.73 $\pm$ 0.08 d	0.36 $\pm$ 0.01 cd
<i>DXS1</i> -4	2.12 $\pm$ 0.05 ab	0.35 $\pm$ 0.01 d
<i>DXS1</i> -5	1.94 $\pm$ 0.11 bc	0.43 $\pm$ 0.02 a
<i>DXS1</i> -6	2.13 $\pm$ 0.10 a	0.34 $\pm$ 0.05 d
<i>DXS1</i> -7	2.12 $\pm$ 0.13 ab	0.35 $\pm$ 0.04 cd
<i>DXS1</i> -8*	2.21 $\pm$ 0.14 a	0.37 $\pm$ 0.03 bcd
<i>DXS1</i> -9*	2.01 $\pm$ 0.20 abc	0.42 $\pm$ 0.03 ab
<i>DXS4</i> -1	1.74 $\pm$ 0.15 c	0.33 $\pm$ 0.02 a
<i>DXS4</i> -2	1.79 $\pm$ 0.15 c	0.34 $\pm$ 0.03 a
<i>DXS4</i> -3	1.94 $\pm$ 0.05 bc	0.34 $\pm$ 0.00 a
<i>DXS4</i> -4	2.17 $\pm$ 0.18 a	0.37 $\pm$ 0.04 a
<i>DXS4</i> -5*	2.16 $\pm$ 0.10 a	0.35 $\pm$ 0.03 a
<i>DXS4</i> -6*	2.14 $\pm$ 0.07 ab	0.38 $\pm$ 0.01 a
<i>DXS6</i> -1	1.45 $\pm$ 0.03 b	0.27 $\pm$ 0.01 c
<i>DXS6</i> -2	1.90 $\pm$ 0.05 a	0.37 $\pm$ 0.01 a
<i>DXS6</i> -3	1.46 $\pm$ 0.02 b	0.32 $\pm$ 0.00 b
<i>DXS6</i> -4	1.94 $\pm$ 0.19 a	0.32 $\pm$ 0.01 b
<i>DXS6</i> -5	1.89 $\pm$ 0.10 a	0.37 $\pm$ 0.01 a
<i>DXS6</i> -6	1.41 $\pm$ 0.12 b	0.28 $\pm$ 0.03 c
<i>DXS6</i> -7	1.52 $\pm$ 0.01 b	0.31 $\pm$ 0.00 b
<i>DXS6</i> -8*	1.82 $\pm$ 0.12 a	0.37 $\pm$ 0.02 a
<i>DXS6</i> -9*	1.87 $\pm$ 0.19 a	0.37 $\pm$ 0.02 a

dependent (Harborne and Williams, 2002); thus, the variability in monoterpene content among the spike lavender plants analyzed reflects the bulked seed origin of the plants used as a source of explants for transformation. It is worth noting that the most common compounds found in the oils from transgenic and control spike lavender oils, namely, α - and β -pinene, cineole, camphor, linalool, α -terpineol, and borneol, were within the ranges observed in other studies on chemical composition of spike lavender oils (Asociación Española de Normalización y Certificación, 1997; Harborne and Williams, 2002; Salido et al., 2004). As already suggested (Dudareva et al., 2004), we found that the formation of volatile compounds in spike lavender is spatially regulated, with flowers producing the most diverse and the highest amount of these compounds. The spatial regulation of essential oils is especially striking for linalool, a major constituent of the oil from flowers that is present in trace amounts in the oil from leaves. Constitutive expression of *DXS* should supply linalool precursors to both leaves and flowers. Although the higher increase in essential oil production in transgenic plants was observed in leaves, their linalool content did not increase. These results support the idea that, besides precursor availability, spatial

regulation of enzyme activities is necessary for the production of linalool in spike lavender leaves.

Gene expression variability and silencing are major problems in the production of stable transgenic plants (Kohli et al., 2003). Because of this, the stable expression of foreign genes and their Mendelian segregation must be corroborated. These studies are difficult in most long-lived perennials like spike lavender. Two years after their transfer to the greenhouse, most of the spike lavender flowered. Thus, we undertook the characterization of the progeny resulting from controlled self-pollination of T_0 lines. Results from PCR and Southern-blot analyses revealed Mendelian segregation ratios of the *DXS* transgene. Also, T_1 progeny that inherited the *DXS* transgene had significantly enhanced essential oil yield, and a positive correlation was quite evident between monoterpene content and *DXS* transgene expression in leaves. It is now well accepted that single-copy lines are predisposed to stability, whereas multicopy lines are prone to instability over successive generations (Meyer and Saedler, 1996). Results herein demonstrate that the high yielding monoterpene phenotype was inherited in progeny of both single- and two-copy lines of the *DXS* transgene.

Metabolic engineering of volatile terpenoids can impose a cost on plant growth and fitness, caused not only by the reduced supply of precursors to branching (primary metabolites) pathways but also by the toxicity of the resulting compounds to plant cells (Aharoni et al., 2005, and refs. therein). Aromatic species like spike lavender, with specialized secretory structures for trafficking and storage of these metabolites, might avoid any detrimental effect related to toxicity, but unforeseen alterations in the level of important terpene primary metabolites cannot be ruled out. Because of this, we analyzed chlorophyll and carotenoid content in transgenic progenies that did or did not inherit the *Arabidopsis DXS* gene. Because the *DXS* transgene was constitutively overexpressed, a general increase of those terpenoids derived from the MEP pathway should be expected. In fact, the overexpression of *DXS* in *Arabidopsis* produced increases in various isoprenoids, including total chlorophylls and carotenoids (Estévez et al., 2001). However, the overexpression of a bacterial *DXS* gene under the control of the CaMV 35S promoter in transgenic tomato lines did not modify the carotenoid content in either leaves or fruits (Enfissi et al., 2005). *DXS* overexpression did not increase chlorophyll and carotenoid content in spike lavender, and some of the transgenic plants showed a reduced level of these plastid-produced metabolites. These results suggest that constitutive *DXS* overexpression does not necessarily mean the same *DXS* activity in all the plant organs, tissues, and even cellular types. As demonstrated by Guevara-García et al. (2005), the MEP pathway is regulated at both transcriptional and posttranscriptional (translational and/or posttranslational) levels. Thus, *DXS* activity might be finely regulated at

the cellular level. This argument would explain why DXS overexpression in spike lavender increases essential oil production in leaf and flower glandular trichomes but does not increase carotenoids and chlorophylls in photosynthetic cells. This suggestion does not rule out other possibilities such as a strong sink effect of glandular trichomes, where essential oils are produced, which may limit the availability of substrates for primary metabolites. In this way, a trend to a negative correlation between essential oil yield and either chlorophyll or carotenoid content was observed in DXS progenies. Nevertheless, this trend was only significant for DXS4 progenies ($r = -0.88$, $P = 0.02$ and $r = -0.89$, $P = 0.02$, for essential oil yield and chlorophyll or carotenoid content, respectively). Note, however, that the increased monoterpene phenotype of transgenic spike lavender did not apparently affect plant development and fitness.

In conclusion, this study demonstrates the potential of the first step of the MEP pathway as a site for metabolic engineering. To our knowledge, results herein represent one of the first examples of an aromatic crop in which end-product monoterpenes can be elevated by increasing the supply of precursors to specific branches of the isoprenoid pathway. Given the economic importance of spike lavender for fragrance, flavor, and pharmaceutical industries, results reported here can be of value to improve and shorten the breeding programs of the species. Besides the biotechnologically relevant enhancement in the yield of spike lavender oil, plants stably overexpressing DXS provide a valuable model for studying the monoterpene formation pathway and its regulatory mechanism.

MATERIALS AND METHODS

Plant Material and Bacterial Strain

Bulked seeds of spike lavender (*Lavandula latifolia* Medicus) from Spanish natural populations (Intersemillas SA) were germinated under sterile conditions as described by Calvo and Segura (1988). The first two pairs of leaves from 40-d-old seedlings were used as primary explants for transformation.

Transgenic T_0 lines refer to plants regenerated from explants originally infected with *Agrobacterium tumefaciens*. T_1 plants (first generation) are seed-derived plants obtained from controlled self-pollination of T_0 plants. Non-transgenic, wild-type spike lavender plants were grown under the same conditions as controls. Both flowers and either developing (first and second verticils) or fully expanded (fourth to 10th verticils) leaves were sampled for molecular and phenotypical analyses.

Agrobacterium strain C58, containing the plasmid pLBI1DXSBS1, kindly provided by Dr. Boronat (University of Barcelona), was used for transformation experiments. This binary vector contains a *nptII* marker gene, driven by the nopaline synthase promoter and terminator, and the full-length open reading frame from the *Arabidopsis* (*Arabidopsis thaliana*) DXS cDNA (AT4G15560), under the control of the CaMV 35S promoter and nopaline synthase terminator.

Spike Lavender Transformation

Agrobacterium-mediated transformation and regeneration of spike lavender was carried out according to Nebauer et al. (2000). Regenerated plants were transplanted to 100-mL pots containing a medium of perlite:peat moss (1:1, v/v). Once acclimatization to ex vitro conditions was accomplished, plants were transferred to the greenhouse with natural light (200–600 μmol

$\text{m}^{-2} \text{s}^{-1}$) and photoperiod and definitively transplanted to 10-L pots containing the same substrate. Dripping irrigation provided moisture for maintenance of vigorous growth. The pots were regularly surface irrigated with half-strength Hoagland and Arnon (1950) nutrient solution.

After 2 years under greenhouse conditions, all transgenic T_0 lines flowered. Progenies were obtained by selfing (July–August) in controlled conditions. T_1 seeds were harvested in October, germinated in vitro as described by Calvo and Segura (1988), and seedlings transplanted to pots and transferred to the greenhouse as previously described. Leaves of T_1 seedlings were used to evaluate segregation of DXS and *nptII* genes by PCR analysis.

PCR and Southern Blotting

DNA was isolated from leaves (50–100 mg fresh weight) of in vitro-grown transgenic and wild-type plants using the cetyl trimethyl ammonium bromide (CTAB) procedure described by Doyle and Doyle (1990). For large-scale leaf preparations (2 g fresh weight from greenhouse-grown plants), genomic DNA was extracted as follows. Leaf material was ground to a powder in a mortar and pestle with liquid nitrogen; the powder was transferred to a centrifuge tube and incubated at 60°C for 1 h with 10 mL of the CTAB isolation buffer (2% [w/v] CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl, pH 8.0, 1% [w/v] polyvinylpyrrolidone-40, and 0.2% [v/v] 2-mercaptoethanol). Then, 10 mL chloroform:isoamyl alcohol (24:1, v/v) was added, vortexed 10 s, and centrifuged at 4°C for 20 min at 8,000 rpm. Then, 1 mL of preheated (60°C) 10% (w/v) CTAB was added to the supernatant and incubated at 60°C for 2 min. Afterward, 20 mL of MilliQ water was added, the extract incubated at 4°C for 1 h, and centrifuged at 4°C for 20 min at 12,000 rpm. The pellet was resuspended in 2.5 mL of Tris/EDTA 1 M NaCl, vortexed 10 s with 2 mL phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), and centrifuged at room temperature for 20 min at 3,000 rpm. Nucleic acids were precipitated at 4°C for 20 min by adding 2 volumes of cold 96% (v/v) ethanol and then centrifuged at 4°C for 20 min at 13,000 rpm. The pellet, previously washed in 70% ethanol and dried at room temperature, was finally resuspended in 300 μL of MilliQ water with RNase.

PCRs were performed in 50- μL reaction volumes containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM MgCl_2 , 20 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1 mM dNTP, 0.25 mM each oligonucleotide primer, and 4 units of Taq polymerase (Biotools). Primer sets used were as follows: 5'-GTCGCTTGGTCCGTCATTTCG-3' and 5'-GTCATCTCACCTTGCTCCTGCC-3' for the *nptII* gene, and 5'-GTTCA-TTTCATTGGAGAGGAC-3' and 5'-TGGGAATTGTTGTGGGTTC-3' for the DXS gene. The predicted sizes of the amplified DNA fragments were 526 bp and 320 bp for *nptII* and DXS, respectively. Amplification parameters for the *nptII* gene were 95°C for 5 min followed by 40 cycles of 95°C for 1 min and 65°C for 2 min. A 65°C incubation for 5 min as final step was included. Amplification parameters for the DXS gene were 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, with a final extension step of 72°C for 7 min. Amplified products were detected by UV light fluorescence after electrophoresis on 1% (w/v) agarose Tris-borate/EDTA gels with 1.27 μM ethidium bromide.

The Southern-blot analysis was performed with nonradioactive digoxigenin-11-dUTP labeled probes. DNA from each sample (20–30 μg) was individually digested with 240 units of *EcoRI* for 16 h at 37°C and subsequently separated on 1% (w/v) agarose Tris-borate/EDTA gel at 60 V for 6 to 8 h. The gel was incubated for 30 min in 0.25 N HCl, twice for 30 min in 0.5 N NaOH, 1.5 M NaCl, and twice for 30 min in 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl. DNA was transferred onto positively charged nylon membranes (Boehringer Mannheim) by capillary blotting (Sambrook et al., 1989). DNA was bound to the membranes by UV crosslinking. Membranes were prehybridized as specified by the manufacturer (Boehringer Mannheim) in a hybridization oven (Ecogen SRL) for 3 h at 60°C. Probes used for detecting *nptII* and DXS transgenes were obtained by PCR amplification as previously described, but without dNTP and using 100 ng of the plasmid as a template and 0.1 mM of the PCR DIG Labeling mix (Roche Applied Science). Hybridization was carried out overnight at 60°C in hybridization buffer containing 20 ng/mL of the probe, followed by two nonstringent and two stringent washes ($2 \times \text{SSC}/0.1\%$ [w/v] SDS and $0.1 \times \text{SSC}/0.1\%$ [w/v] SDS, respectively). Detection of the digoxigenin-hybridized fragments was carried out according to the manufacturer's recommendations.

Northern Blotting

Total RNA was extracted from 0.8 g of fresh, fully expanded leaves and 0.4 g flowers of transgenic and wild-type plants by using the Tripure Isolation

Reagent (Roche Applied Science) according to the supplier's protocol. RNA concentrations were determined by measuring A_{260} . For RNA gel-blot analysis, 30 μ g of total RNA was denatured for 15 min at 65°C in loading buffer (1.25 $\times$ MAE, 55% [v/v] formamide, 7.4% [v/v] formaldehyde, 3.2% [v/v] glycerol, and 0.05% [w/v] bromophenol blue) prior to electrophoresis. RNA was separated at 60 V for 4 h on 1% (w/v) agarose gel in MAE containing 2.2% (v/v) formaldehyde and transferred by capillary action onto Hybond-N nylon membranes (Amersham Pharmacia). DNA probes, labeled with [α - 32 P]dCTP, were prepared by random priming of the fragments amplified by PCR. The *DXS* fragment (561 bp) was amplified from the plasmid with primers 5'-GAAACCCAACAACAATTCCC-3' and 5'-GGCTTCATAAGCCTGTCC-TGCCG-3', whereas the tubulin3 (*TUB3*) fragment (969 bp), used to verify the equal loading of RNA in the gel slots, was amplified from Arabidopsis cDNA, with primers 5'-CCTGATACTTCGTCTTTGGTCAATCC-3' and 5'-GAACTCCATCTCGTCCATTCC-3'. For both genes, the amplification parameters were 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min. A final step of 72°C for 7 min was included. Blots were prehybridized in buffer (0.4 M NaH_2PO_4 , pH 7.2, 1 mM EDTA, and 7% [w/v] SDS) at 65°C for 20 min and hybridized overnight at 65°C in the same buffer with 5 ng/mL of the corresponding probe. The membranes were washed twice for 10 min in 4 $\times$ SSC, 0.1% (w/v) SDS at 65°C, twice for 5 min in 0.4 $\times$ SSC, 0.1% (w/v) SDS at 65°C, and exposed to an autoradiographic film at -80°C.

Western Blotting

The polyclonal antibody Ab-AtDXS was generated in a rabbit injected with the synthetic peptide GPMHQLAAKVDV, corresponding to positions G296 to V307 of the Arabidopsis DXS protein. Both the peptide and the antibodies were ordered from Bio-Synthesis and produced in their facilities. For western blots, crude protein extracts from wild-type Arabidopsis var. Columbia and young spike lavender leaf tissues (0.5 g) were obtained by harvesting in liquid nitrogen and grinding in 1 mL of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.5, 15 mM MgCl_2 , 100 mM KCl, 0.25 mM Suc, 10% [v/v] glycerol, 1% [w/v] polyvinylpyrrolidone-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.4% [v/v] 2-mercaptoethanol) with 33.33 μ L of the Protease Inhibitor Cocktail (Sigma, P-9599), followed by two successive centrifugations (25,000 rpm for 30 min at 4°C). Protein samples were quantified with Bradford reagent (Bio-Rad) using bovine serum albumin as a standard, and then 30 μ g of total proteins was separated in 8% SDS-PAGE. To verify equal protein loading, a parallel gel was run and stained with Coomassie Brilliant Blue R-250 (Sigma) and destained with 40% (v/v) methanol and 10% (v/v) acetic acid. The proteins were electrotransferred onto Immune-Blot polyvinylidene difluoride membranes (Bio-Rad) using the Mini Tran-Blot Cell (Bio-Rad) for 16 h at 40 V with the transfer buffer (10 mM NaHCO_3 , 3 mM Na_2CO_3 , and 20% [v/v] methanol). Blots were blocked in PBS buffer and 0.1% (v/v) Tween 20 (PBS-T) with 5% (w/v) nonfat dry milk for 1 h. Incubation with the anti-serum (1:2,000) in PBS-T was carried out for 1 h at room temperature. Blots were then washed three times for 5 min in PBS-T and incubated for 1 h with peroxidase-conjugated secondary antibody diluted 1:7,500 in PBS-T. After three washes in PBS-T, cross-reacting bands were detected using the ECL Western-Blotting Analysis System kit (Amersham Biosciences).

Essential Oil Analysis

Leaves and inflorescences from each examined plant were treated separately for essential oil extraction, and the tissue was manually crushed and mixed to ensure sample uniformity. Air-dried (for 30 d), fully expanded leaves (1.5 g) or flowers (0.5 g of cymes with three to five open flowers) were distilled in 100 mL of water in a Clevenger-type apparatus for 1.5 h, containing *n*-tetradecane and naphthalene as internal standards. Oils obtained were recovered with hexane, dried over anhydrous sodium sulfate, filtered through 0.22 μ m polyvinylidene difluoride Millipore membranes, and adjusted to a final volume of 10 or 50 mL with hexane to obtain 10 μ g/mL *n*-tetradecane and 400 μ g/mL naphthalene or 2 μ g/mL *n*-tetradecane and 80 μ g/mL naphthalene for leaves or flower distillates, respectively. Oils were kept in airtight glass containers at 4°C until further use.

Qualitative essential oil analysis was accomplished by GC and GC/MS. GC analyses were performed with a Focus GC (Thermo Finnigan) equipped with a flame ionization detector and fitted with a BP-20 capillary column (polyethylene glycol, 30-m $\times$ 0.25-mm $\times$ 0.25- μ m film; SGE Europe), carrier gas He at 1 mL/min, and 1.5 μ L was injected in splitless mode (0.8 min) with

an AI 3000 Autosampler (Thermo Finnigan). The normal oven temperature was programmed initially at 40°C for 1 min, followed by a ramp of 4°C/min to 130°C, and finally held isothermal at 130°C for 25 min. Temperatures of injector and detector were 230°C and 260°C, respectively. To separate (-)-trans-pinocarveol and (1S)-cis-verbenol, the temperature was programmed at 40°C for 1 min, followed by a ramp of 2°C/min to 58°C, a second ramp of 1°C/min to 70°C, held isothermal at 70°C for 1 min, followed by a third ramp of 2°C/min to 130°C, and finally held isothermal at 130°C for 15 min. GC/MS analyses were carried out on an Agilent 6890N gas chromatograph with an HP-INNOWax Column (Agilent Technologies) similar to the previously used BP-20; the coupled mass spectrometer was an Agilent 5973N with a quadrupole mass selective detector. All mass spectra were acquired in the electron impact mode at 70 eV. The mass spectrometer scanned in the range of 30 to 550 mass-to-charge ratio at a rate of 5.36 scans/s. The other analytical conditions were the same as for GC analysis. Compounds were identified by comparison of retention indices and mass spectra to those of authentic standards, or by reference mass spectra in a computer library (Wiley7n). MS/GC was performed by the Central Service for the Support to Experimental Research (SCIE, University of Valencia).

The products were quantified (milligram per gram tissue dried) by comparison of detector response with that of the internal standards, assuming equal response factors. Also, percentages of compounds were determined from their peak areas. The relative peak area for individual constituents was determined using the Chrom-Card S/W program (Thermo Finnigan). All analyses were performed at least four times.

Chlorophyll and Carotenoid Content

Extraction and determination of total chlorophylls and carotenoids were conducted as described by Lichtenthaler (1987). Extracts were obtained in 100% acetone from 200 to 300 mg of fresh, fully developed leaves from spike lavender plants that did or did not inherit the *DXS* gene. Spectrophotometric quantifications were carried out in a Shimadzu UV-1203 spectrophotometer. All analyses were performed at least three times.

Statistical Analyses

Significance of the variation in essential oil production and chlorophyll and carotenoid content between transgenic and control plants was determined using ANOVA (Statgraphics Plus for Windows version 2.1), and mean comparisons using Tukey's (1953) procedure were carried out when appropriate. Inheritance observed data were compared to the expected ratios using a chi-squared analysis with Yates's correction (Zar, 1996).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number U27099.

Received July 6, 2006; accepted September 13, 2006; published September 15, 2006.

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