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Enhanced levels of S-linalool by metabolic engineering of the terpenoid pathway in spike lavender leaves

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ABSTRACT

Transgenic *Lavandula latifolia* plants overexpressing the linalool synthase (*LIS*) gene from *Clarkia breweri*, encoding the LIS enzyme that catalyzes the synthesis of linalool were generated. Most of these plants increased significantly their linalool content as compared to controls, especially in the youngest leaves, where a linalool increase up to a 1000% was observed. The phenotype of increased linalool content observed in young leaves was maintained in those T_1 progenies that inherit the *LIS* transgene, although this phenotype was less evident in the flower essential oil. Cross-pollination of transgenic spike lavender plants allowed the generation of double transgenic plants containing the *DXS* (*1-deoxy-D-xylulose-5-P synthase*), coding for the first enzyme of the methyl-D-erythritol-4-phosphate pathway, and *LIS* genes. Both essential oil yield and linalool content in double DXS-LIS transgenic plants were lower than that of their parentals, which could be due to co-suppression effects linked to the structures of the constructs used.

1. Introduction

Lavandula latifolia Medicus (spike lavender), of the Lamiaceae family, is a perennial evergreen shrub up to 100 cm with a flowering peak in June–September. The species, of great economic interest, is cultivated worldwide for the production of a commercially valuable essential oil, which mostly contains monoterpenes, the C10 class of the isoprenoids (Harborne and Williams, 2002; Muñoz-Bertomeu et al., 2007a). As in other Lamiaceae, spike lavender essential oil is produced in specialized glandular trichomes found on the surface of leaves and flowers, although oil yield and composition are different between the two tissues (Muñoz-Bertomeu et al., 2007a).

Monoterpenes are crucial for many biological activities of plants, such as defense against herbivores and pathogens, allelopathic interactions and pollination (Baldwin et al., 2006; Dudareva et al., 2006; Langenheim, 1994; Pichersky and Gershenzon, 2002; Tholl, 2006). Monoterpenes are also widely used in perfumery, cosmetics, food processing and therapeutics (Lubbe and Verpoorte, 2011; Verlet, 1993; Woronuk et al., 2011). Because of the implication of monoterpenes in many biological functions and their economic value, the isoprenoid pathway has been a focus for many scientists (Roberts, 2007).

The production of monoterpenes begins with the synthesis of the universal terpene precursors isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), mainly through the plastidial methyl-D-erythritol 4-phosphate (MEP) pathway (Dudareva et al., 2005; Lange and Turner, 2013). Nevertheless, the classic mevalonate (MVA) pathway could also provide IPP precursors for monoterpene biosynthesis in spike lavender (Muñoz-Bertomeu et al., 2007b). IPP and DMAPP are then condensed head-to-tail by geranyl diphosphate synthase (GPPS) to produce the linear monoterpene precursor geranyl diphosphate (GPP; C10), which is subsequently transformed into various monoterpenes by specific enzymes collectively known as monoterpene synthases (Degenhardt et al., 2009; Fig. 1).

Metabolic engineering aimed at monoterpene production has become an intensive research topic in recent years (Dong et al., 2013; Farhi et al., 2011; Houshyani et al., 2013; Lange and Ahkami, 2013). The simplest approach to produce aromatic plants that potentially accumulate elevated levels of a specific monoterpene is

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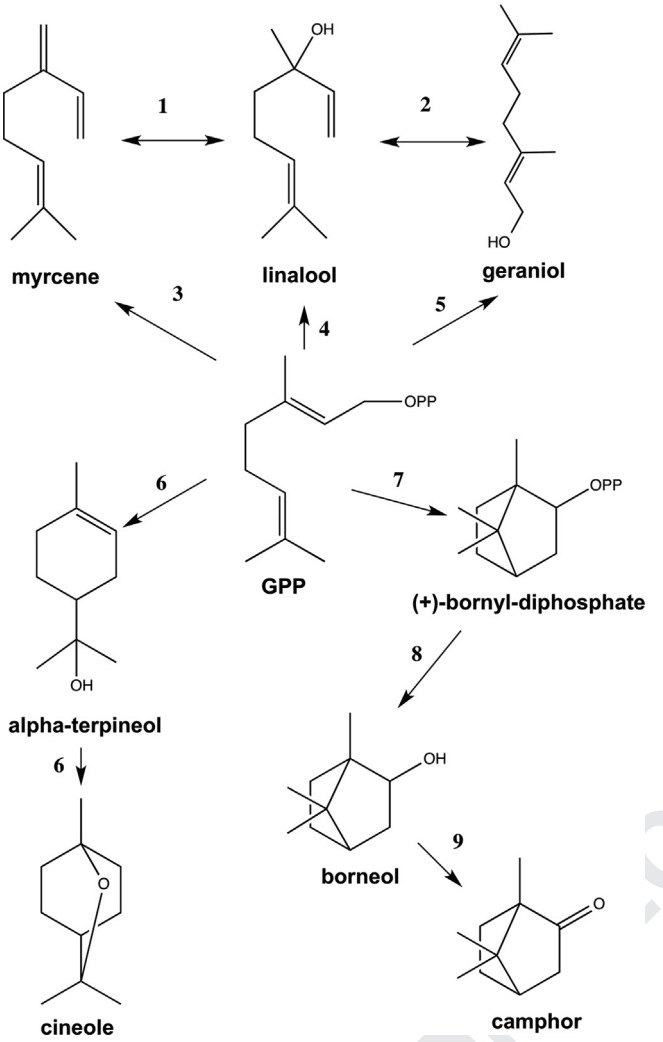

Fig. 1. Scheme for the synthesis of the monoterpenes myrcene, linalool, geraniol, alpha-terpineol, borneol, cineole and camphor. GPP, geranyl diphosphate; 1, linalool dehydratase–hydrolase; 2, geraniol hydroxymutase; 3, myrcene synthase; 4, S-linalool synthase; 5, monoterpenyl-pyrophosphatase; 6, cineole synthase; 7, bornyl diphosphate synthase; 8, bornyl diphosphate diphosphatase; 9, borneol dehydrogenase.
Adapted from Hallahan and Croteau (1989), Hallahan (2000), Dewick (2002) and Brodkorb et al. (2010).

the overexpression of a particular terpene synthase gene. To date, this approach has been employed, in a few lamiaceae such as peppermint (*Mentha* × *piperita*), cornmint (*Mentha arvensis*) and spike lavender but with different success. Thus, the overexpression of the spearmint (*Mentha spicata*) limonene synthase (*MsLS*) gene in mint species did not lead to substantive changes on oil composition (Diemer et al., 2001; Krasnyanski et al., 1999; Mahmoud et al., 2004). In contrast, the constitutive overexpression of the same gene in spike lavender, led to a significant increase in the accumulation of limonene in the leaf essential oil, where this monoterpene is a minor component (Muñoz-Bertomeu et al., 2008). A developmental time course study indicated that young leaves of transgenic spike lavender plants contained increased limonene content (more than 450% increase compared to controls) that correlated with the highest transcript accumulation of the *MsLS* gene (Muñoz-Bertomeu et al., 2008).

The monoterpene profile of spike lavender essential oil differs from that of the most valuable common lavender (*Lavandula angustifolia* Mill.) by higher relative amounts of camphor and cineole (at the expense of linalool and linalyl acetate), which results in a more pungent scent (Lis-Balchin, 2002). It is known that the relative concentration in both camphor and linalool determines the quality and prize of the essence. The most appreciated oils for the perfume and cosmetic industries are those with high content in linalool and low content in camphor, while those richer in camphor are mainly used in aromatherapy and phytotherapy (Kaloustian et al., 2000). Thus, from an economical point of view, the biotechnological breeding of spike lavender should be addressed to improve the quality of the essential oil by increasing the ratio of linalool to camphor. Because of this, we studied whether the heterologous expression of the linalool synthase (LIS) enzyme, catalyzing the synthesis of linalool from GPP (Lavy et al., 2002; Fig. 1), might modify the monoterpene profile of spike lavender. To this end, transgenic plants overexpressing the *LIS* cDNA from *Clarkia breweri* were generated. In other species such as *Dianthus caryophyllus* (Lavy et al., 2002), *Solanum lycopersicum* (Lewinsohn et al., 2001), *Petunia hybrida* W115 (Lücker et al., 2001) and *Saccharomyces cerevisiae* (Herrero et al., 2008) *LIS* overexpression increased production of linalool and/or their derivatives (trans-linalool, 8-hydroxylinalool and S-linalyl-β-D-glucopyranoside respectively). As a final approach to improve both quantity and quality of the spike lavender essential oil, we also generated, by controlled crosses, transgenic plants bearing two of the genes involved in the first and last steps of linalool biosynthesis. Specifically, we obtained double-transgenic plants containing the *DXS* (*1-deoxy-D-xylulose-5-P synthase*) gene, coding for the first enzyme of the MEP pathway and the *LIS* gene. We previously demonstrated that the constitutive overexpression of an Arabidopsis *DXS* cDNA in spike lavender led to the most highly increased essential oil yields reported thus far (up to 359% in leaves and up to 74% in flowers) without negative effects on oil composition (Muñoz-Bertomeu et al., 2006).

2. Material and methods

2.1. Plant material

Bulked seeds of *Lavandula latifolia* Medicus (spike lavender), from Spanish natural populations (Intersemillas SA, Valencia Spain), were germinated under sterile conditions as described by Calvo and Segura (1988). The first two pairs of leaves from 40-day 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 either controlled self- or cross-pollination of T_0 plants. Non-transgenic, wild type (WT) spike lavender plants were grown under the same conditions.

Both flowers and either pooled leaves from 4th to 10th whorl or single cohorts of leaves at five stages of development were sampled for molecular and phenotypical analyses. The single cohorts of leaves were sampled at the 1st whorl with fully open leaves (average length 1.3 cm; hereafter referred to as stage a), 2nd whorl (2.8 cm; stage b), 3rd and 4th whorls (4.8 cm; stage c), 5–7th whorls (5.3 cm; stage d), and 8–10th whorls (5.5 cm; stage e).

2.2. Vector construct

The 2.76 kb cDNA from *Clarkia breweri* (A. Gray) Greene that contains the coding sequence for *LIS* gene was provided by Professor Pichersky (Department of Molecular, Cellular, and Developmental Biology, University of Michigan) and received in *pBluescript II SK* (+). This plasmid was digested with *Sal*I to achieve linear DNA and, using the Klenow enzyme, non-cohesive ends

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were produced. The DNA was then digested with *Dra*I and *Bam*HI enzymes producing a 2.76 kb fragment containing the *LIS* gene. This fragment was inserted into the plasmid pBI121, from which the *gus*A (β-glucuronidase) gene had been eliminated with *Bam*HI and *Ecl*136II to produce a 11.1 kb fragment that was separated by electrophoresis in 1 × TBE (90 mM boric acid, 90 mM Tris–HCl pH 8.0 and 2 mM of EDTA) 0.8% agarose and purified with QIAquick Gel Extraction Kit following manufacturer instructions. The new binary vector, named pBILIS, contained the *npt*II (neomycin phosphotransferase II) marker gene, driven by the nopaline synthase promoter and terminator, and the LIS-cDNA, under the control of the CaMV35S (cauliflower mosaic virus) promoter and nopaline synthase terminator. It was introduced in *Escherichia coli* strain XL1Blue according to the technique described by Sambrook and Russell (2001). Finally, and using the freeze–thaw method (Holsters et al., 1978), the binary vector pBILIS was transferred into the *A. tumefaciens* strain C58.

2.3. Spike lavender transformation

Agrobacterium-mediated transformation and regeneration of spike lavender was carried out according to Nebauer et al. (2000). Briefly, after leaf coculture with the bacteria, regenerated transformed shoots were selected on a modified Murashige and Skoog (1962) medium supplemented with BA (Bencil-adenine), IAA (indole acetic acid), and kanamycin. Primary transformed shoots were elongated, rooted and transferred to soil. Acclimatization and maintenance of regenerated plants under greenhouse conditions were accomplished as previously described (Muñoz-Bertomeu et al., 2006). After two years, some of the transgenic T_0 lines flowered. Progenies of these T_0 lines 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 *LIS* and *npt*II genes by PCR analysis.

Cross-pollinations were also performed between transgenic spike lavender lines for the genes *DXS* (Muñoz-Bertomeu et al., 2006) and *LIS* genes maintained in the greenhouse. Line DXS6 was used as pollen donor plant. The corresponding recipient transgenic lines were LIS1, LIS2 and LIS8. In late October, mature fruits from crosses were collected. Subsequently, T_1 seeds were germinated and handled as previously described.

2.4. PCR and southern blotting

DNA was isolated as described in Muñoz-Bertomeu et al. (2006). 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 $(NH_4)_2SO_4$, 0.1 mM dNTP, 0.25 mM of each oligonucleotide primer, 100 ng DNA, and 4 units of Taq polymerase (Biotools, Madrid, Spain). Primer sets used were: 5′-GTCGCTTGGTCGGTCATTTCG-3′ and 5′-GTCATCTCACCTTGCTCCTGCC-3′ for the *npt*II gene, and 5′-GGGAGGAAGTTGATGAGAAGAAGC-3′ and 5′-CTTGTTAACCCCTTTC CCCAC-3′for the *LIS* gene. The predicted sizes of the amplified DNA fragments were 526 bp and 1329 bp for *npt*II and *LIS*, respectively. Amplification parameters for both genes were 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C, 2 min at 60 °C and 2 min at 72 °C, and finally 7 min at 72 °C. The amplification products of DNA were separated by electrophoresis in 1X TBE gels with 1% agarose and 0.05 mg mL^{-1} of ethidium bromide.

The Southern Blot analyses for *DXS* and *npt*II genes were performed with non-radioactive digoxigenin-11-dUTP labeled probes as described in Muñoz-Bertomeu et al. (2006). The same protocol was employed for the *LIS* gene, but using *Bam*HI as endonuclease. Probe used to detect *LIS* transgene was obtained by PCR amplification using the above cited primers.

2.5. Northern blotting

Total RNA extraction, electrophoresis and subsequent transference onto Hybond-N nylon membranes (Amershan Pharmacia) were performed as described in Muñoz-Bertomeu et al. (2006). DNA probes, labeled with [α-^{32}P]dCTP, were prepared by random priming of the fragments amplified by PCR. The *LIS* fragment was amplified from the plasmid with primers and PCR conditions above indicated, whereas the *α3-tubulin* fragment (969 bp), used to verify the equal loading of RNA in the gel slots, was amplified from *Arabidopsis thaliana* cDNA as previously described (Muñoz-Bertomeu et al., 2006). 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 or 55 °C for 20 min, and hybridized overnight at 65 °C or 55 °C in the same buffer with 5 ng mL^{-1} of the *LIS* or *TUB3* probes, respectively. The membranes were washed twice for 10 min in 4 × SSC, 0.1% (w/v) SDS at 65 °C, twice for 5 min in 0.4 × SSC, 0.1% (w/v) SDS at 65 °C and exposed to an auto-radiographic film at −80 °C.

2.6. Essential oil analysis

Air dried (for 30 days) pooled leaves from 4th to 10th verticils (1.5 g) or flowers (0.5 g) from each examined plant were hydrodistilled as described in Muñoz-Bertomeu et al. (2006). Also, fresh leaves (0.1 g) from each of the five developmental stages previously indicated were transferred to 20-mL glass tubes containing 4 mL hexane with n-tetradecane and naphthalene as internal standards. Small glass beads (0.1 g; 425–600 μm in diameter, Sigma) were incorporated into the mixture before shaking up in a vortex for 1 min. After 4 h incubation at room temperature, the extract was filtered through Millipore membranes (0.22 μm) and concentrated under a nitrogen stream to 1 mL. Essential oil quantification was performed by gas chromatography (GC) and GC/mass spectrometry (MS) analyses as described in Muñoz-Bertomeu et al. (2006).

2.7. Statistical analysis

Significance of the variation in essential oil production between transgenic and control plants was determined using analysis of variance (Statgraphics Plus for Windows version 2.1, Manugistics, Rockville, MD), 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 square analysis with Yates's correction (Zar, 1996).

3. Results

3.1. Generation and evaluation of transgenic plants overexpressing the LIS gene

Spike lavender transformation was achieved after the Nebauer et al. (2000) protocol using *A. tumefaciens* strain C58 harboring the plasmid pBILIS containing the *Clarkia breweri LIS* and the *neomycin phosphotransferase* II (*npt*II) genes. All 26 kanamycin-resistant plants were first screened by PCR for the presence of *npt*II and *LIS* genes (data not shown). All 14 *npt*II$^+$/*LIS*$^+$ plants were cloned, acclimatized to ex vitro conditions, and transferred to the greenhouse for further analyses. Eight putatively independent primary transformants (T_0), designed as LIS1 to LIS8, were obtained. All the transgenic lines were morphologically indistinguishable from the control plants.

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The number of *LIS* transgene inserts in the 8 transgenic lines was determined by Southern Blot analysis. As shown in Fig. 2, the eight transgenic lines displayed different hybridization patterns indicating independent transformation events. Specifically, lines had one (LIS1 and LIS4), two (LIS5 and LIS6), three (LIS7 and LIS8) or four (LIS2 and LIS3) inserts of the *LIS* transgene. Identical hybridization patterns were obtained for the *npt*II transgene (data not shown), corroborating the insertion of the complete T-DNA.

Northern Blot analyses were first performed in leaves from the first and second whorls of all T_0 transgenic and control plants (developmental stages a and b; see Material and methods). *LIS* mRNA was detected in all lines but the control. Irrespective of the transgenic line, the level of transcripts drastically decreased in the second whorl in comparison with the first one. In this whorl the higher expressions were found in lines LIS2, LIS3, LIS7 and LIS8 (Fig. 3).

The expression of the *LIS* transgene was also determined in cohorts of leaves sampled at 5 different developmental stages (a–e; see Material and methods) of lines LIS3 (with 4 inserts of the transgene) and LIS4 (with 1 insert). Transgene expression in both lines was dependent on the developmental stage of the leaves, with the youngest (developmental stage a) showing the highest expression; no *LIS* gene signal was detected in controls at any of the leaf developmental stages tested (Fig. 4).

3.2. Overexpression of the LIS gene increases linalool content in the essential oil from spike lavender leaves

S-linalool synthase catalyzes the conversion of GPP to linalool (Fig. 1). This reaction has been shown to be the source of linalool in plants (Lücker et al., 2001). Nevertheless, in the bacteria *Castellaniella defragrans*, linalool can be also formed from other monoterpenes (Fig. 1), such as myrcene and geraniol (Brodkorb et al., 2010). Because of this, we first quantified these three compounds as well as other quantitatively important monoterpenes such as cineole and camphor. The essential oil yield was also determined.

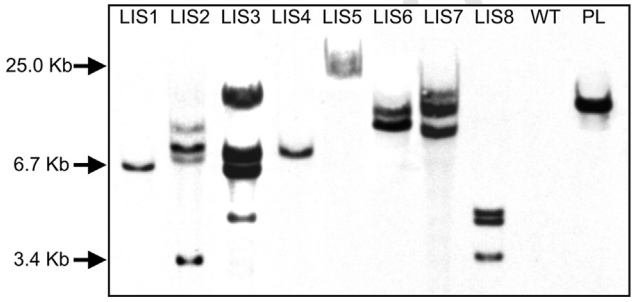

Fig. 2. Southern Blot hybridization analysis of the *LIS* transgene in spike lavender T_0 lines (LIS1–LIS8); WT, wild type plant; PL, plasmid pBILIS.

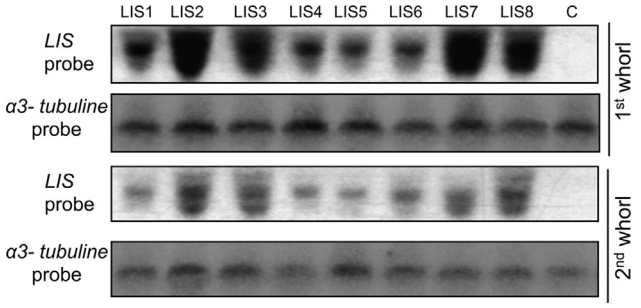

Fig. 3. Expression analysis of the *LIS* transgene in leaves from the first and second whorl of control (C) and the eight transgenic T_0 spike lavender lines (LIS1–LIS8). The expression of the *α3-tubuline* gene is shown to certify equal loading.

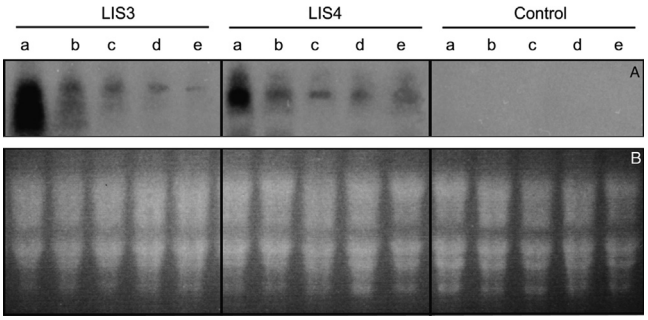

Fig. 4. Expression analysis of the *LIS* gene during leaf development in transgenic T_0 spike lavender lines LIS3 and LIS4. (A) Transcript accumulation in leaves at five developmental stages (a–e) from transgenic and control plants. (B) Gel loading control for the Northern Blot.

Table 1
Content (microgram per gram fresh weight) of Myrcene, Linalool, and Geraniol in hexane-extracted essential oil from leaves (whorl 1 and 2) of control and transgenic T_0 spike lavender plants transformed with the *Clarkia breweri LIS* gene. All reported values represent the mean ± SD of at least 3 measurements. For each column and whorl, mean values followed by the same letter are not significant different according to Tukey's test at $P \leq 0.05$.

Whorl	Line	Myrcene		Linalool		Geraniol	
1	LIS1	72.80 ± 0.20	d	8.70 ± 0.10	d	2.30 ± 0.00	cd
	LIS2	79.90 ± 0.90	c	10.10 ± 0.10	c	2.90 ± 0.00	ab
	LIS3	55.8 ± 0.50	f	57.10 ± 0.20	a	1.10 ± 0.1	e
	LIS4	116.10 ± 4.40	a	61.70 ± 1.70	b	3.10 ± 0.10	a
	LIS5	63.30 ± 1.10	e	6.30 ± 0.00	e	1.90 ± 0.20	d
	LIS6	37.80 ± 3.10	g	3.60 ± 0.00	g	0.70 ± 0.10	e
	LIS7	93.80 ± 0.20	b	5.10 ± 0.00	f	2.70 ± 0.30	bc
	LIS8	79.80 ± 0.60	c	6.70 ± 0.00	e	3.10 ± 0.20	a
	Control	61.20 ± 0.10	ef	4.90 ± 0.00	f	2.20 ± 0.00	d
2	LIS1	21.60 ± 0.40	ef	2.10 ± 0.00	de	0.70 ± 0.00	de
	LIS2	38.80 ± 1.60	b	3.00 ± 0.10	de	1.20 ± 0.10	cd
	LIS3	19.30 ± 0.60	f	45.70 ± 0.20	b	0.40 ± 0.00	e
	LIS4	51.30 ± 1.20	a	33.40 ± 1.10	a	3.00 ± 0.10	b
	LIS5	12.50 ± 2.10	g	1.40 ± 0.00	e	0.60 ± 0.10	e
	LIS6	15.00 ± 0.40	g	3.00 ± 0.00	de	1.30 ± 0.00	c
	LIS7	25.20 ± 0.60	de	2.90 ± 0.10	de	6.10 ± 0.50	a
	LIS8	33.50 ± 2.20	c	5.40 ± 0.10	c	5.70 ± 0.10	a
	Control	26.50 ± 0.80	d	3.60 ± 0.00	d	1.60 ± 0.00	c

In transgenic spike lavender plants overexpressing the limonene synthase gene, the high amount of limonene was detected in developing leaves (Muñoz-Bertomeu et al., 2008). Then, the first series of essential oil analyses were performed in leaves from the first and second whorls of the 8 LIS transgenic lines and controls. GC and GC/MS determinations allowed the identification of 19 constituents, accounting for 95–97% of the oil. In all transgenic and control lines, the content in linalool, myrcene and geraniol was higher in leaves from the first whorl, which positively correlate with the *LIS* mRNA levels in these leaves (Table 1 and Fig. 3). Linalool production in leaf oils of spike lavender significantly increased (from 100% to 1000%) in 7 out of the 8 transgenic lines as compared to controls; these increases were particularly striking for lines LIS3 (10.8- and 12.8-fold in the first and second whorl, respectively) and LIS4 (12.7- and 9.4-fold in the first and second whorl, respectively). There was not a clear correlation between linalool and myrcene or geraniol contents, being difficult to establish whether these monoterpenes could contribute to linalool production in spike lavender (Table 1).

Based on the above results, analysis of essential oil content in the transgenic lines LIS3 and LIS4 was extended to leaves sampled at five (a–e) developmental stages. Essential oil yield, linalool, myrcene and geraniol, as well as camphor and cineole, the main

contributors to the spike lavender leaf essential oil, were quantified (Table 2).

In both transgenic and control lines, the essential oil yield and the amount of the analyzed monoterpenes, but geraniol in the transgenic lines, decreased with leaf development; this trait was more evident after the leaves reached 3 cm in length (stage c; Table 2).

A clear correlation between overexpression of *LIS* transgene and essential oil yield could not be established. In contrast, the effect of this transgene on linalool content was evident at all leaf developmental stages, although the highest amounts, as compared to control, were obtained in leaves at developmental stages a, b and c (Table 2). Furthermore, Northern Blot analyses at the different developmental stages indicated that the increases in the expression of the *Clarkia LIS* gene paralleled the increase in the linalool content of the transgenic spike lavender LIS3 and LIS4 (Fig. 3 and Table 2). The effect of *LIS* transgene expression on myrcene and geraniol was line-dependent; thus, and as stated before, our results do not support another biosynthetic pathways for linalool than the activity of the linalool synthase enzyme in spike lavender.

Transgenic LIS3 and LIS4 lines showed increased or reduced amounts in camphor and cineole, respectively, as compared to control. Since the content of these two main constituents of the spike lavender essential oil is genotype dependent (Harborne and Williams, 2002; Muñoz-Bertomeu et al., 2007b), it is difficult to attribute this pattern to a direct effect of the transgene.

Table 2
Effect of leaf developmental stage (LDS) on essential oil yield and production (microgram per gram fresh weight) of representative monoterpenes in hexane-extracted essential oil of transgenic T_0 spike lavender lines LIS3, LIS4 and Control. All reported values represent the mean ± SD of at least 3 measurements. Within each LSD and monoterpene, values followed by the same letter are not significant different according to Tukey' test at $P \leq 0.05$.

LSD	Line	Myrcene	1,8-Cineole	Camphor
a	LIS3	55.77 ± 0.53b	3401.82 ± 5.38b	4898.74 ± 4.59b
	LIS4	116.11 ± 4.41a	3221.82 ± 4.34c	5883.03 ± 2.07a
	Control	61.86 ± 2.84b	6334.05 ± 7.98a	1705.60 ± 2.00c
b	LIS3	19.30 ± 0.64c	2020.87 ± 0.83c	3172.85 ± 3.42b
	LIS4	51.30 ± 1.20a	2748.97 ± 12.75b	4719.92 ± 2.94a
	Control	39.68 ± 0.63b	7042.11 ± 7.48a	1699.93 ± 0.42c
c	LIS3	29.43 ± 0.53a	584.73 ± 0.27b	601.17 ± 0.81a
	LIS4	4.13 ± 0.09c	245.94 ± 0.30c	337.36 ± 0.36b
	Control	15.35 ± 0.37b	1552.12 ± 3.98a	313.50 ± 0.62c
d	LIS3	8.33 ± 0.77b	177.74 ± 1.00b	149.35 ± 0.20b
	LIS4	3.12 ± 0.02c	146.01 ± 0.33c	215.35 ± 1.31a
	Control	10.44 ± 0.23a	391.30 ± 1.18a	38.40 ± 0.37c
e	LIS3	4.18 ± 0.31b	74.86 ± 0.69c	65.53 ± 0.41b
	LIS4	3.87 ± 0.13b	190.03 ± 0.19b	316.67 ± 2.02a
	Control	6.19 ± 0.08a	237.67 ± 0.02a	16.22 ± 0.06c
LSD	Line	Linalool	Geraniol	Oil Yield
a	LIS3	57.13 ± 0.18b	1.12 ± 0.12c	9695.96 ± 5.81c
	LIS4	61.69 ± 1.66a	3.18 ± 0.10b	10,671.89 ± 18.25a
	Control	6.15 ± 0.17c	3.73 ± 0.14a	9284.74 ± 12.47b
b	LIS3	45.72 ± 0.20a	0.38 ± 0.03c	5985.21 ± 3.76c
	LIS4	33.41 ± 1.12b	2.95 ± 0.05b	8686.82 ± 23.78b
	Control	6.19 ± 0.08c	3.44 ± 0.00a	10,036.50 ± 3.36a
c	LIS3	16.89 ± 0.18a	0.83 ± 0.03b	1578.44 ± 2.69b
	LIS4	5.55 ± 0.19b	2.17 ± 0.03a	7,05.10 ± 0.69c
	Control	1.51 ± 0.03c	0.64 ± 0.01c	2178.86 ± 4.11a
d	LIS3	14.25 ± 0.07a	0.92 ± 0.04b	469.76 ± 1.67b
	LIS4	1.18 ± 0.00b	2.25 ± 0.12a	427.80 ± 0.57c
	Control	0.60 ± 0.01c	0.61 ± 0.01c	573.72 ± 0.80a
e	LIS3	8.32 ± 0.09a	0.67 ± 0.00b	210.25 ± 0.04c
	LIS4	1.91 ± 0.02b	3.43 ± 0.09a	571.07 ± 2.10a
	Control	0.35 ± 0.01c	0.49 ± 0.03c	337.07 ± 0.34b

3.3. *The phenotype of increased linalool content observed in young leaves was maintained in those T_1 progenies that inherit the LIS transgene*

Self-pollination of transgenic T_0 LIS plants was performed for two consecutive years. Seeds produced from lines LIS3, LIS4, LIS6 and LIS8 were germinated in vitro (Supplementary Table S1). Subsequently, seedlings were grown aseptically, analyzed for the inheritance of the transgene, acclimatized under controlled conditions for two months and transferred to the greenhouse.

The inheritance of the *LIS* transgene was analyzed by PCR. Most of the seedlings inherited the transgene (Fig. 5A), but only line LIS6 produced enough plantlets to accurately study this inheritance. The chi-squared analysis (Table 3) demonstrated that the LIS6 mother plant, that had two inserts of the *LIS* gene, showed a 3:1 segregation, suggesting that in this plant the two inserts were integrated in the same chromosome; thus *LIS* transgene behaves as typical dominant, linked gene. Southern Blot analysis showed that LIS^+ T_1 plants inherited the two copies of the transgene, corroborating this hypothesis (Fig. 5B). Twenty-seven plants were acclimatized and used for further analyses.

Initially, linalool content in hexane-extracted essential oil from leaves sampled at the five developmental stages detailed above was determined in five randomly chosen T_1 plants (LIS6-12, LIS6-14, LIS6-21, LIS6-28, and LIS6-30). As shown in Fig. 6A, the temporal linalool accumulation pattern observed in leaves from the LIS6 T_0 mother plant was retained in those progenies that inherited the transgene. These plants also had elevated linalool phenotype as compared to their T_1 counterpart (LIS6-12) that did not inherit the *LIS* gene. The positive effect of *LIS* transgene on linalool production was more noticeable when the percentage of linalool content over the total essential oil was determined. In that case, all lines that inherited the *LIS* transgene showed an increased proportion of linalool at all leaf developmental stages (Fig. 6B).

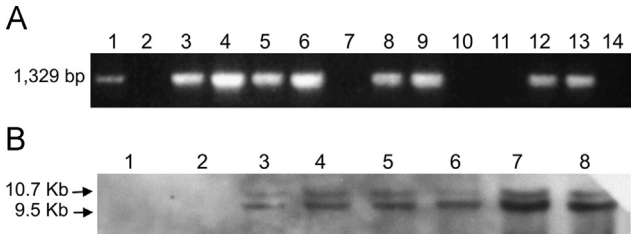

Fig. 5. Molecular analyses of the progeny from transgenic spike lavender LIS6 line. (A) PCR analysis of the *LIS* gene: Lane 1, pBILIS plasmid; Lane 2, non-transgenic plant; Lane 3, parental T_0 LIS6; Lanes 4–14, LIS6 progeny. (B) Southern blotting of the *LIS* gene; Lane 1, control; Lane 2, LIS6-12 T_1 plant that did not inherit the *LIS* transgene; Lanes 3–7, selected progenies of LIS6 plant that inherited the *LIS* transgene; Lane 8, parental T_0 LIS6.

Table 3
Segregation of *LIS* gene in T_1 LIS6 plants from self-pollinated transgenic T_0 lines of spike lavender plants. LIS^+, LIS^- = PCR-positive and PCR-negative, respectively. A Chi-square value > 3.84 indicates a significant deviation from the expected ratio (P = 0.05).

Ratio	Observed		Expected		Chi-square value
	LIS^+	LIS^-	LIS^+	LIS^-	3:1 (1 insert)
3:1	22	5	20.25	6.75	0.61
15:1	22	5	25.30	1.68	6.83

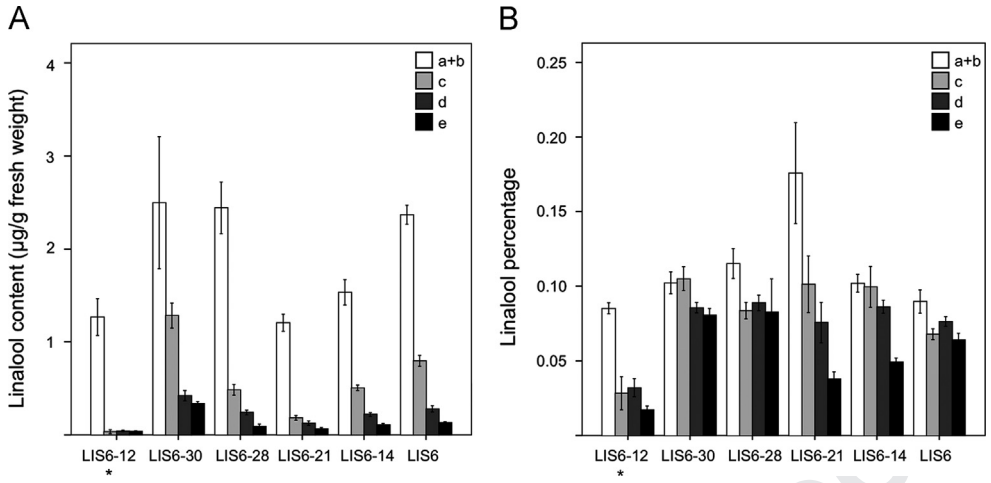

Fig. 6. Effect of leaf developmental stage (LDS) on the linalool content in hexane-extracted essential oils from transgenic T_0 and T_1 spike lavender plants. (A) Linalool content (microgram per gram of fresh weight). (B) Linalool percentage. All reported values represent the mean ± SE of at least 3 measurements. *T_1, plant that did not inherit the *LIS* transgene.

Table 4
Essential oil yield and production (microgram per gram dried weight) of representative monoterpenes from hydrodistilled leaves (4–10th whorls) of transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic LIS6 line. All reported values represent the mean ± SD of at least 3 measurements. For each column, mean values followed by the same letter are not significant different according to Tukey' test at $P \leq 0.05$. #, T1 plant that did not inherit the *LIS* transgene.

Line	Myrcene	1,8-cineole	Camphor	Linalool	Geraniol	Oil yield
LIS6-12 #	1.20 ± 0.17d	5047 ± 179c	3891 ± 255d	0.68 ± 0.04d	1.59 ± 0.11d	9046 ± 321d
LIS6-14	3.56 ± 0.23b	6786 ± 51a	4563 ± 231c	1.24 ± 0.07b	1.84 ± 0.21c	11,513 ± 225c
LIS6-21	0.43 ± 0.02e	1928 ± 114d	3446 ± 418e	0.61 ± 0.06d	1.42 ± 0.24de	5446 ± 540e
LIS6-28	4.94 ± 0.77a	6927 ± 146a	8467 ± 307a	1.60 ± 0.06a	3.17 ± 0.15a	15,622 ± 317a
LIS6-30	0.52 ± 0.03e	6108 ± 186b	2735 ± 118f	0.91 ± 0.04c	1.19 ± 0.07e	8951 ± 306d
LIS6	2.14 ± 0.64c	6758 ± 366a	5970 ± 256b	1.19 ± 0.03b	2.36 ± 0.15b	12,923 ± 296b

Table 5
Essential oil yield and production (milligram per gram dried weight) of representative monoterpenes from hydrodistilled flowers of transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic LIS6 line. All reported values represent the mean ± SD of at least 3 measurements. For each column, mean values followed by the same letter are not significant different according to Tukey's test at $P \leq 0.05$. Control: pooled flowers from LIS6-12 and LIS6-26 that did not inherit the LIS transgene.

Line	Myrcene	1.8-Cineole	Camphor	Linalool	Geraniol	Oil yield
Control	0.86 ± 0.26bc	13.17 ± 1.60ab	12.89 ± 2.17cd	11.31 ± 1.46bcd	0.09 ± 0.01cd	47.62 ± 1.48bcd
LIS6	0.59 ± 0.13bcd	14.51 ± 1.40a	12.67 ± 0.72cd	8.82 ± 0.47d	0.10 ± 0.02bcd	44.76 ± 1.16cd
LIS6-13	0.39 ± 0.02cd	12.72 ± 0.96ab	12.87 ± 0.36cd	9.76 ± 0.73cd	0.09 ± 0.01cd	44.38 ± 1.37cd
LIS6-14	0.40 ± 0.05cd	13.07 ± 0.39ab	10.60 ± 0.32d	8.99 ± 0.39d	0.09 ± 0.01cd	41.71 ± 1.51d
LIS6-21	0.80 ± 0.11bcd	11.95 ± 0.39abc	23.25 ± 0.43a	14.17 ± 1.79ab	0.13 ± 0.00a	61.28 ± 0.79a
LIS6-25	1.06 ± 0.05ab	11.55 ± 0.19bc	20.35 ± 0.54a	15.82 ± 1.84a	0.14 ± 0.01ab	60.09 ± 2.48a
LIS6-28	0.37 ± 0.06d	8.02 ± 0.52d	17.03 ± 1.23b	13.43 ± 1.03abc	0.11 ± 0.01ab	49.06 ± 2.78bc
LIS6-29	0.68 ± 0.15bcd	9.15 ± 0.89cd	09.90 ± 0.87d	15.04 ± 2.46a	0.08 ± 0.02d	42.66 ± 4.93d
LIS6-34	1.49 ± 0.30a	14.50 ± 1.19a	14.02 ± 0.26bc	11.55 ± 0.47bcd	0.09 ± 0.00cd	51.65 ± 1.84b

Since the commercial spike lavender oil is obtained from hydrodistillation, essential oil analyses were also accomplished in hydrodistillates of pooled air-dried leaves (whorls 4–10th) and flowers from T_0 LIS6 and their progenies.

Data from hydrodistillated leaves (Table 4 and Supplementary Table S2) corroborated those previously obtained using the direct hexane extraction method (Fig. 6, A and B) related to the increased linalool content in T_1 lines that inherited the transgene. Note that the essential oil yield as well as the content of the other analyzed terpenes (myrcene, cineole, geraniol and camphor) were line dependent and were not affected by the presence of the transgene.

As expected, flowers produced more essential oil than leaves (Tables 4 and 5). The effect of *LIS* transgene on essential oil composition was less evident in flowers since only two out of the 8 analyzed T_1 lines that inherited the transgene (LIS6-25 and LIS6-29) produced significantly more linalool than their counterparts that did not inherit it (Table 5). However, a significant linalool percentage increase in essential oil from flowers was only observed in T_1 LIS6-29 line (Supplementary Table S3).

3.4. Double transgenic spike lavender plants containing the DXS and LIS transgenes had neither increased leaf linalool content nor essential oil yield

Plants were obtained from seeds recovered after controlled crosses between T_0 DXS and LIS spike lavender lines. Plants resulting for these crosses were cloned in vitro, acclimatized to ex vitro conditions and transferred to the greenhouse.

Crosses between DXS6 (Muñoz–Bertomeu et al., 2006) and LIS1, LIS2 or LIS8 transgenic lines produced 20 seeds, although only 10 of them yielded viable offspring (Supplementary Table S4). PCR analyses showed that 2 plants (DXS6-LIS8-1 and DXS6-LIS8-2)

inherited both *DXS* and *LIS* genes (Fig. 7). Further Southern Blot analysis (Fig. 8) showed that DXS6-LIS8-1 plant inherited one of the two inserts of the *DXS* gene and the 3 inserts of the *LIS* gene, whereas DXS6-LISI8-2 plant inherited the two inserts of *DXS* gene and the insert of *LIS* gene. Line DXS6-LIS8-3 did not inherit any of the genes and consequently was used as internal control (IC) in further experiments.

Expression levels of *DXS* and *LIS* transgenes from the double transgenic (DXS6-LIS8-1 and DXS6-LIS8-2) plants were determined by Northern Blot analyses. Fig. 9 shows the *LIS* and *DXS* transcript levels in leaves from the 1–3rd and 4–10th whorls of all double transgenic, DXS6 and negative control plants. DXS6 mRNA was detected in all lines, except the controls. Double transgenic lines showed a lower level of *DXS* transcripts than the parental DXS6, especially in the older whorls (4–10th). This also holds true for the *LIS* gene, although in this case the transcript level reduction was even more dramatic as compared to the *LIS* expression level in T_0 LIS8 parental plant (Fig. 3). These results suggest the occurrence of a generalized transcript suppression process affecting both *DXS* and *LIS* genes of these double transgenic plants.

Essential oil analyses by direct extraction in hexane were performed in cohorts of leaves sampled at the developmental stages a+b, c and d. In all cases, DXS6 parental and progenies that did not inherit any of the genes were used as controls. As expected, and irrespective of the presence of the transgenes, leaves from the youngest whorls produced more essential oil (Supplementary Fig. S1) and linalool (Fig. 10). Transgenic plants containing one (*DXS*) or two (*DXS* and *LIS*) genes produced more essential oil than non-transgenic controls (Supplementary Fig. S1). None of those plants that inherited both genes produced a specific linalool increase (Fig. 10).

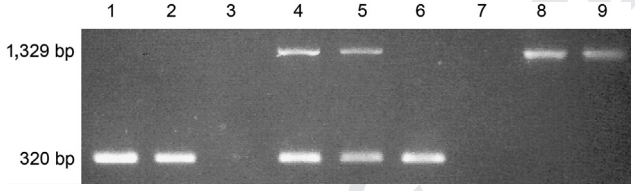

Fig. 7. PCR analysis of *DXS* and *LIS* genes in progenies from crosses between transgenic DXS and LIS spike lavender plants. Lane 1, *DXS* plasmid; Lane 2, Parental DXS6; Lane 3, wild type plant; Lanes 4–6, Progenies from DXS6 × LIS8 crosses; Lane 7, wild type plant; Lane 8, Parental LIS8; and Lane 9, *LIS* plasmid.

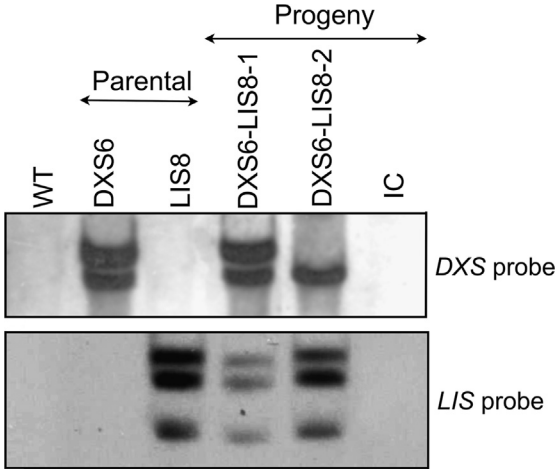

Fig. 8. Southern blotting analysis of *DXS* and *LIS* genes in progenies from crosses between transgenic DXS and LIS spike lavender plants. IC, internal control (progeny that did not inherit any of the genes); WT, wild type plant.

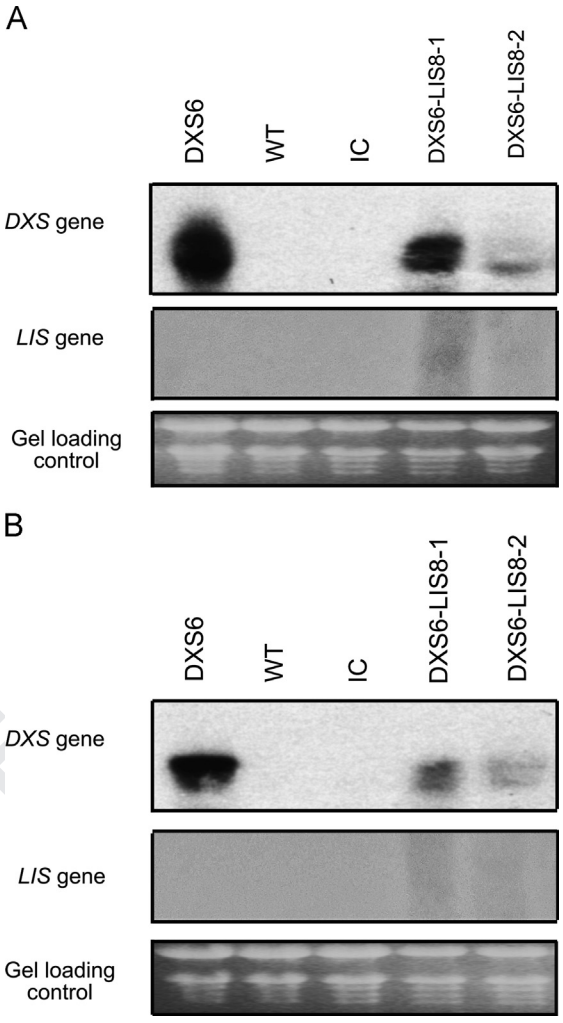

Fig. 9. Expression analysis of the *DXS* and *LIS* transgenes in leaves from 1th to 3th (A) and 4th to 10th (B) whorls of double transgenic (DXS6-LIS8-1 and DXS6-LIS8-2), DXS6 parental and control spike lavender plants; WT, wild type plant; IC, internal control (progeny that did not inherit any of the genes). The loading control for the Northern blotting is also showed.

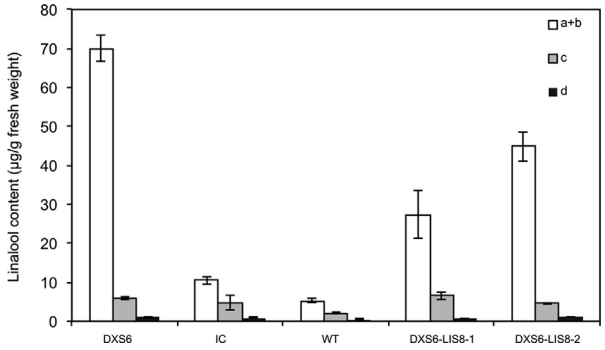

Fig. 10. Effect of leaf developmental stage (LDS a+b, c and d) on Linalool content (microgram per gram of fresh weight) in DXS6 parental and double transgenic DXS6-LIS8 plants. IC, internal control (progeny that did not inherit any of the genes). WT, wild type plant. Reported values represent the mean ± SD of three measurements.

4. Discussion

The monoterpene synthases catalyze the first committed step that leads to the synthesis of the different monoterpene families (Bohlman

et al., 1998). Because of this, the manipulation of the gene expression of monoterpene synthases might be a fundamental tool to modify the profile of the essential oil. This strategy has been successfully used to provide the capability for the synthesis of monoterpenes to plants and/or tissues that do not naturally do so (Aharoni et al., 2005; Degenhardt et al., 2003; Lewinsohn et al., 2001; Wu et al., 2006). To date, however, this strategy has been employed with variable success in Lamiaceae; thus, whereas in mints the overexpression of the *MsLS* gene did not produce substantive variations on oil composition (Diemer et al., 2001; Krasnyanski et al., 1999; Mahmoud et al., 2004), in spike lavender the overexpression of the same transgene lead to a significant increase of limonene (Muñoz-Bertomeu et al., 2008). Linalool is one of the monoterpenes that determines more directly the commercial value of the spike lavender essential oil. Therefore, the availability of spike lavender plants with an enhanced production of linalool does have a potential industrial interest.

Following the *Agrobacterium* transformation protocol described for the species (Nebauer et al., 2000), eight T_0 spike lavender lines overexpressing the *LIS* gene from *Clarkia breweri* were produced. Most of these T_0 plants showed a significant increase in linalool content as compared to control. The effect of *LIS* transgene was particularly striking in the youngest leaves of 2 lines where a linalool increase up to a 1000% was observed. *LIS* gene was driven by a constitutive promoter (CaMV35S), but their transcript abundance was dependent on the developmental stage of the leaf, reaching a maximum in the youngest leaves, which correlated with its higher linalool content. Linalool accumulation in *L. angustifolia* was also correlated with transcript levels for endogenous *LIS* gene (Lane et al., 2010). *LIS* transcripts and linalool content decreased along with the developmental stage of the leaf, but the increase in linalool content was maintained at all developmental stages in transgenic plants as compared to control. This high linalool producing phenotype was maintained in leaf essential oil of the progenies that inherited the transgene. This phenotype was, however, less evident in the essential oil from flowers of these progenies. The most important difference between leaf and flower spike lavender oil is the amount of linalool (traces in leaves and more than 15% of the total oil in flowers), which suggest a strong spatial regulation of the LIS enzyme as reported for other monoterpene synthases and plant species (Dudareva et al., 2004; Irmisch et al., 2012; Tholl, 2006). Thus, it seems that the overexpression of *LIS* transgene does not increase linalool content in organs with a high endogenous LIS activity (flowers) as it does in those with less activity (leaves), which is in accordance with previous studies in other model plants, like *Dianthus caryophyllus* (Lavy et al., 2002), *Solanum lycopersicum* (Lewinsohn et al., 2001) and *Petunia hybrida* W115 (Lücker et al., 2001). In the case of *Dianthus caryophyllus*, the headspace GC/MS analyses showed emission of linalool and its derivatives, cis- and trans-linalool oxide. In contrast, the analysis of flower extracts of the transgenic plants produced an enhanced amount of trans-linalool oxide but not linalool, revealing that the oxidation of linalool might be a way to store this compound (Lavy et al., 2002). Glycosylation of linalool, which converts it into a non-volatile form, was the reason for the lack of linalool emission in transgenic petunia overexpressing the *LIS* gene (Lücker et al., 2001). In the case of tomato fruits, not only linalool was found but also its derivative 8-hydroxylinalool (Lewinsohn et al., 2001). All of these data support the idea that linalool might be converted to another compound in order to store it or as a side effect of the normal metabolism of monoterpenes in plants. This might explain the differences observed between the leaves and flowers in spike lavender. A limitation due to the viability of IPP might also be the cause for it in the case of flowers.

Overexpression of *DXS* transgene in spike lavender leads to increased linalool content in essential oil from flowers but not from leaves (Muñoz-Bertomeu et al., 2006). 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. Based on this idea we hypothesized that the generation of spike lavender plants overexpressing both *DXS* and *LIS* genes would be an appropriate strategy to increase linalool content in leaf essential oil. In the present work, we report the generation of these double transgenic plants by controlled pollination. The obtained results show, however, that double transgenic plants did not increase linalool content in leaves and produced less essential oil than their DXS6 mother plant. Northern Blot analyses show a decrease in the level of transcripts of both genes. Then, transgene suppression processes could explain the decreased amount of essential oil found in double transgenic plants, as has been already observed in many plants. It is known that transgenes can be silenced by the host plant RNA silencing machinery (Baulcombe, 2004; Kalantidis et al., 2006; Kanazawa, 2008; Vaucheret, 2006). The RNA silencing machinery evolved as a defense mechanism against transposable elements, viruses and other forms of foreign nucleic acids (Baulcombe, 2005; Wang and Metzlaff, 2005). Why transgenes activate this silencing machinery is not yet fully understood, although transcripts from genes like *LIS* and *DXS* transgenes, with no CAP structure or poly(A) tail, could have a trigger function (Gazzani et al., 2004; Luo and Chen, 2007; Wassenegger and Krczal, 2006). Also, the lack of introns in the transgene sequence might be a problem (Luo and Chen, 2007; Wassenegger and Krczal, 2006). An intron-less transcript might be processed into dsRNA by the RNA-dependent RNA polymerase 6 (RDR6), and at that point the dsRNA would trigger the RNA silencing machinery. Finally, *DXS* and *LIS* transgenes were under the control of a strong promoter like CaMV35S, whose activity may lead to an accumulation of RNAs that, when exceeds a certain threshold, will activate the plant defense mechanism (Wassenegger and Krczal, 2006). Transgenic *Nicotiana benthamiana* and *N. tabacum* (Dalakouras et al., 2011) expressing a transcriptional fusion of the green fluorescence protein (GFP) cDNA and a 98-bp PSTVd cDNA fragment showed diverse response to spontaneous silencing. In *N. benthamiana*, the self-silencing process involved mRNA degradation and dense DNA methylation of the homologous coding region. In *N. tabacum*, silencing occurred without complete mRNA degradation and with low methylation of the gene region. This might indicate that in plants siRNA-mediated spontaneous silencing might involve translational inhibition in addition to mRNA degradation (Dalakouras et al., 2011). Studies in transgenic Arabidopsis (Luo and Chen, 2007) expressing the *β-glucuronidase* gene in different copy number and arrangements support the positive correlation among transgene copy number, expression, and RDR6 mediated RNA silencing. The transgenic mRNA probably triggers RDR6 by acting as templates for the RNA polymerase.

In conclusion, transgenic spike lavender plants overexpressing the linalool synthase gene from *Clarkia breweri* lead to an increased linalool content in leaves without modifying the total content of essential oil. As remarked, the quality and economical value of spike lavender essential oil depends highly on its percentage of linalool. Therefore, the achievement of plants with an enhancement of this monoterpene production in flowers is highly desirable. We know that this *Clarkia breweri* gene is expressed in spike lavender plants as shown by the results achieved in leaves. Thus, the next step in the production of plants of economic importance would be a new transgenic approach using promoters and terminator specifically addressed to flowers. Also, further studies on the negative effect of the co-expression of *DXS* and *LIS* transgenes in spike lavender essential oil and composition are needed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.03.003.

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