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Expression of spearmint limonene synthase in transgenic spike lavender results in an altered monoterpene composition in developing leaves

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ABSTRACT

We generated transgenic spike lavender (*Lavandula latifolia*) plants constitutively expressing the limonene synthase (*LS*) gene from spearmint (*Mentha spicata*), encoding the LS enzyme that catalyzes the synthesis of limonene from geranyl diphosphate. Overexpression of the *LS* transgene did not consistently affect monoterpene profile in pooled leaves or flowers from transgenic T_0 plants. Analyses from cohorts of leaves sampled at different developmental stages showed that essential oil accumulation in transgenic and control plants was higher in developing than in mature leaves. Furthermore, developing leaves of transgenic plants contained increased limonene contents (more than 450% increase compared to controls) that correlated with the highest transcript accumulation of the *LS* gene. The levels of other monoterpene pathway components were also significantly altered. T_0 transgenic plants were grown for 2 years, self-pollinated, and the T_1 seeds obtained. The increased limonene phenotype was maintained in the progenies that inherited the *LS* transgene.

1. Introduction

Spike lavender (*Lavandula latifolia* Med.), of the family Lamiaceae, is an aromatic shrub native to the Mediterranean region that is cultivated worldwide for oil production. The essential oils that give spike lavender its typical olfactory characteristics are produced in glandular trichomes (Hallahan, 2000) and are primarily composed of monoterpenes (Harborne and Williams, 2002; Muñoz-Bertomeu et al., 2007a), the C10 branch of the isoprenoid family. These volatile lipophilic compounds have a high economic value as flavors, fragrances and medicines (Verlet, 1993). In addition, they play important roles in plant defense, plant-to-plant communication, and pollinator attraction (Dudareva et al., 2006).

Plant isoprenoids are derived from either the mevalonic acid (MVA) pathway, which is active in the cytosol, or from the plastidial 2-methyl-D-erythritol-4-phosphate (MEP) pathway (Rodriguez-Concepción and Boronat, 2002; Fig. 1). Both pathways lead to the formation of the basic terpenoid biosynthesis building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Although there are examples where the MVA and MEP pathways can supply different portions of a molecule, or where there is exchange of common intermediates between the two pathways (Eisenreich et al., 2004; Hampel et al., 2006), it is assumed that monoterpenes are primarily synthesized in the plastids via the MEP pathway-derived IPP and DMAPP (Mahmoud and Croteau, 2002).

With a few exceptions, monoterpene biosynthesis can be divided into four steps (Mahmoud and Croteau, 2002; Dudareva et al., 2004): (1) construction of the basic C5 units (IPP and 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 monoterpene synthases; for this conversion (see Fig. 1), GPP is first ionized and isomerized to produce linalyl diphosphate (LDP), which either produces the acyclic monoterpenes or the α-terpinyl cation, the universal intermediate for cyclic monoterpenes (Bohlmann et al., 1998; Dewick, 2002); and (4) transformation of the parent structures to various derivatives. Fig. 1 summarizes the possible biogenetic routes for the most common plastidial-produced acyclic (myrcene and linalool) and cyclic (limonene, α-pinene, cineole, camphor, α-terpineol and borneol) monoterpenes and the cytosolic sesquiterpenes *t*-caryophyllene and its oxygenated derivative, found in spike lavender oils.

In theory, any of the four steps leading to the biosynthesis of monoterpenes can be engineered in order to increase yield and/or modify the essential oil profile of targeted plants. Thus, manipulation of the steps involved in construction of the basic C5 units resulted in significant increases in essential oils of peppermint (Mahmoud and Croteau, 2001) and spike lavender (Muñoz-Bertomeu et al., 2006) without change in monoterpene composition

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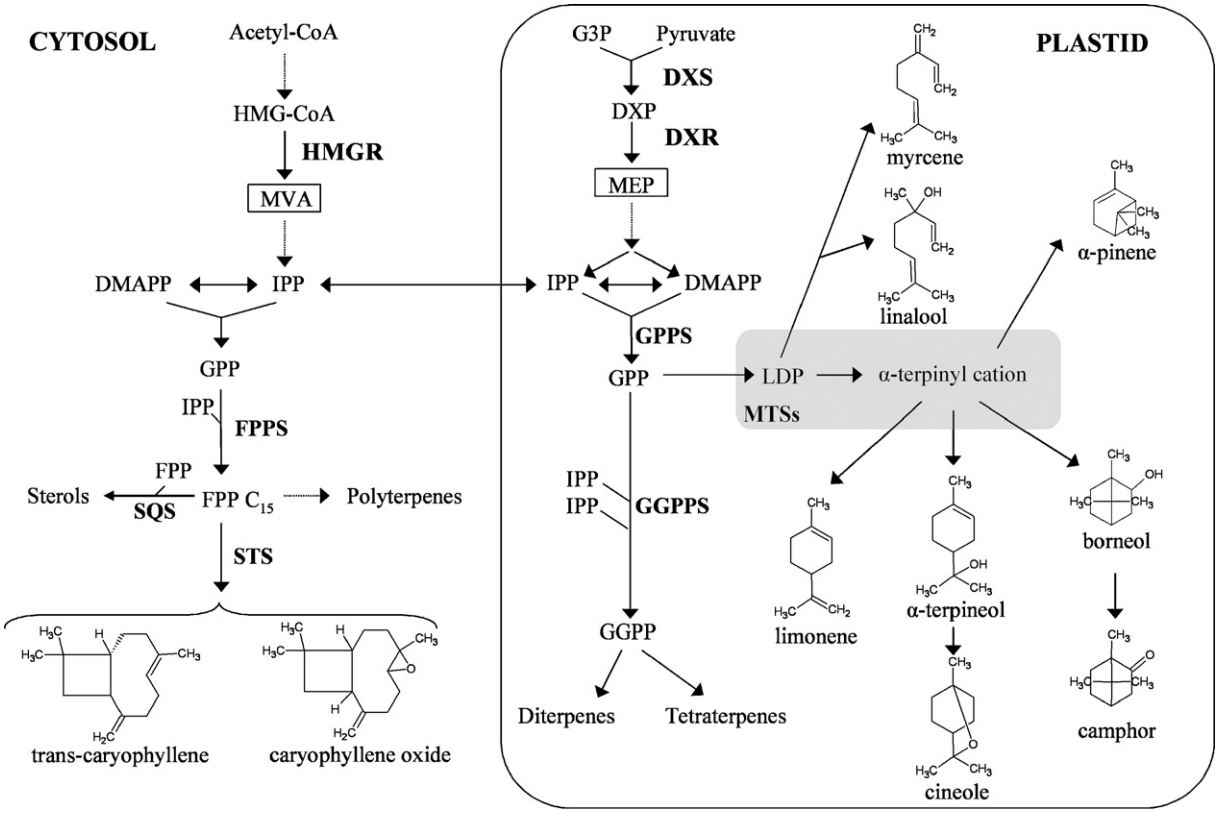

Fig. 1. Isoprenoid biosynthesis in plants. Enzymes are indicated in boldface: DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; GPPS, geranyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl CoA reductase; MTSs, monoterpene synthases; SQS, squalene synthase; STS, sesquiterpene synthase. The first intermediate specific to each pathway is boxed: DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; GPP, geranyl diphosphate; G3P, D-glyceraldehyde-3-phosphate; HMG-CoA, 3-hydroxy-3-methylglutaril-CoA; IPP, isopentenyl diphosphate; LDP, linalyl diphosphate; MEP, methyl-D-erythritol-4-phosphate; MVA, mevalonic acid. The possible biogenetic routes (adapted from Dewick, 2002) and the chemical structures of representative mono- and sesquiterpenes analyzed are shown.

compared with control plants. There are also examples of the production of transgenic plants overexpressing monoterpene synthases, a key control point in the biosynthesis of monoterpenes (Mahmoud and Croteau, 2002). This approach has been successfully undertaken in plants and/or organs that do not naturally produce these compounds (Degenhardt et al., 2003; Aharoni et al., 2005; Wu et al., 2006). To date, examples of the production of transgenic aromatic plants overexpressing monoterpene synthases are scarce and have been focused on mint species transformed with limonene synthase (LS), an enzyme that catalyzes the stereo-specific cyclization of GPP to form the monocyclic monoterpene limonene. This compound has a considerable commercial value in beverages and the cosmetics industry; also (+) limonene has anti carcinogenic properties (Crowell and Gould, 1994; Jun et al., 2006). Krasnyanski et al. (1999) and Diemer et al. (2001) constitutively expressed the spearmint (*Mentha spicata*) *limonene synthase* (*MsLS*) gene in peppermint (*Mentha x piperita*) and cornmint (*Mentha arvensis*). Nevertheless, none of these studies reported substantive effects on oil composition. Recently, Mahmoud et al. (2004) demonstrated that the constitutive expression of *MsLS* in peppermint leaves was insufficient to significantly increase production of the targeted enzyme in the glandular trichomes, where essential oils are biosynthesized, so no influence was observed on the monoterpene profile.

Limonene is a minor constituent of the spike lavender oil (0.5–2%; Asociación Española de Normalización y Certificación, 1997) and so it should be possible to increase its content by metabolic engineering with obvious consequences for the commercial production of this oil. In the present paper, spike lavender was transformed with the *MsLS* gene under the regulation of the CaMV 35S constitutive promoter. Molecular and detailed essential oil analyses of transgenic and control plants revealed important changes in the oil composition as related to leaf development. Our results also demonstrate that the constitutive expression of *LS* gene leads to a change in the monoterpene profile in the youngest leaves.

2. Materials and methods

2.1. Plant material

Bulked seeds of *L. latifolia* Med. (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 controlled self-pollination of T_0 plants. Non-transgenic, wild-type (WT) spike lavender plants were grown under the same conditions as controls.

Both flowers and either pooled leaves from 4th to 10th verticils 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 verticil with fully open leaves (average length 1.3 cm; hereafter referred to as stage I),

2nd verticil (2.8 cm; stage II), 3rd and 4th verticils (4.8 cm; stage III), 5th–7th verticils (5.3 cm; stage IV) and 8th–10th verticils (5.5 cm; stage V).

2.2. Vector construct

The 4S-LS cDNA from spearmint (accession number L13459), kindly provided by Prof. Croteau, Washington State University, Pullman (USA), was received in pBluescript II SK (+), and designated pLC5.2 (Colby et al., 1993). This plasmid was digested with *Bam*HI and *Eco*RV and the 2.4 kb fragment containing the *LS* gene 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. The new binary vector, named pBILS, contained the *npt*II (neomycin phosphotransferase II) marker gene, driven by the nopaline synthase promoter and terminator, and the 4S-*LS*-cDNA, under the control of the CaMV 35S (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 pBILS 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). Acclimatization and maintenance of regenerated plants under greenhouse conditions were accomplished as previously described (Muñoz-Bertomeu et al., 2006). After 2 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 *LS* and *npt*II genes by PCR analysis.

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′-GGTCATCCTTCGTTCTGCGGCTT-3′ and 5′-GCAGAAGGCTCTTGAACTTG-3′ for the *LS* gene. The predicted sizes of the amplified DNA fragments were 526 and 563 bp for *npt*II and *LS*, respectively. Amplification parameters for *npt*II 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 *LS* gene were 94 °C for 2 min followed by 35 cycles of 94 °C for 0.5 min, 60 °C for 0.5 min and 72 °C for 1 min with a final extension step of 72 °C for 5 min. Amplified products were detected by ultraviolet light fluorescence after electrophoresis on 1% (w/v) agarose TBE gels with 1.27 μM ethidium bromide.

The Southern blot analysis was performed with non-radioactive digoxigenin-11-dUTP labeled probes as described in Muñoz-Bertomeu et al. (2006).

2.5. Northern blotting

Total RNA extraction, electrophoresis and subsequent transference onto Hybond–N nylon membranes (Amershan Pharmacia) was 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 *LS* fragment was amplified from the plasmid with primers and PCR conditions above indicated, whereas the tubulin3 (*TUB3*) 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 or 55 °C for 20 min, and hybridized overnight at 65 or 55 °C in the same buffer with 5 ng/mL of the *LS* 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 previously described (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 GC and GC/MS analyses as described in Muñoz-Bertomeu et al. (2006).

2.7. Chlorophyll and carotenoid analysis

Extraction and determination of total chlorophylls and carotenoids were conducted as described in Muñoz-Bertomeu et al. (2006).

2.8. Statistical analysis

Significance of the variation in essential oil production and photosynthetic pigment content 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.

3. Results

3.1. Generation and evaluation of transgenic T_0 spike lavender

Spike lavender transformation was accomplished through an established protocol (Nebauer et al., 2000) using *A. tumefaciens* strain C58 harboring the plasmid pBILS containing spearmint *LS* and *npt*II genes.

The integration of both transgenes in the kanamycin-resistant plantlets was first confirmed by PCR (data not shown). All *LS*$^+$/*npt*II$^+$ plants were cloned, acclimatized to ex vitro conditions and transferred to the greenhouse. Nine putatively independent primary transformants (T_0), designed as LS1–LS9 were obtained. All the transgenic lines were morphologically indistinguishable from the control plants (Supplementary Fig. S.1).

The number of *LS* transgene inserts was determined by Southern blotting of total leaf DNA. As shown in Fig. 2, the nine transgenic lines displayed different hybridization patterns,

indicating independent transformation events. Three out of the nine independent transgenic lines (LS1, LS7 and LS9) presented single copy insertions, whereas the rest of the lines presented from 2 to 11 copies of the *LS* gene (Fig. 2a). The DNA gel blot was subsequently hybridized with the *npt*II probe to verify whether the complete T-DNA was inserted. Hybridization patterns with this probe demonstrated the insertion of the complete T-DNA in lines LS4, LS5 and LS7 (Fig. 2a).

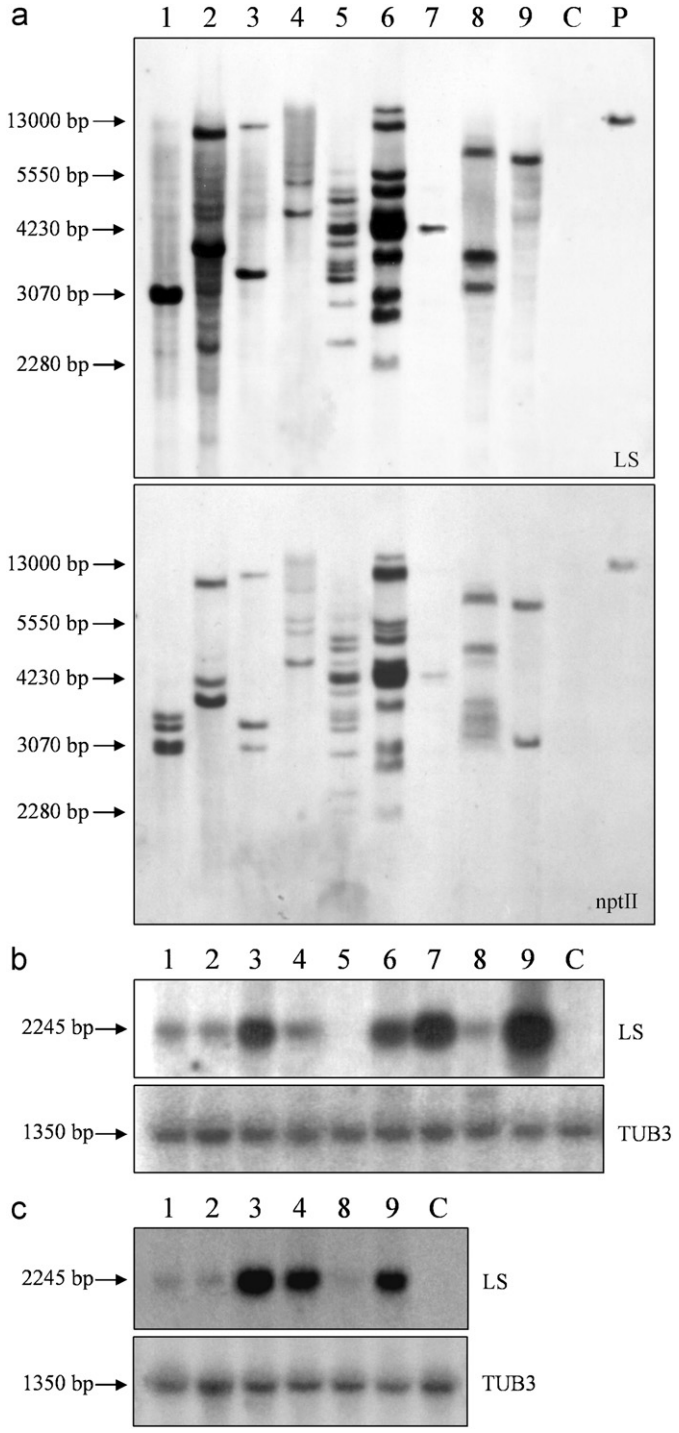

Fig. 2. Molecular analyses of transgenic T_0 spike lavender plants. (a) Southern blot hybridization of *LS* and *npt*II genes. (b and c) RNA gel blot of *LS* transcript accumulation in leaves (b) and flowers (c) of transgenic lines; the expression of the tubulin3 (*TUB3*) gene is shown to verify equal loading. Lane 1, LS1; lane 2, LS2; lane 3, LS3; lane 4, LS4; lane 5, LS5; lane 6, LS6; lane 7, LS7; lane 8, LS8; lane 9, LS9; lane C, control; and lane P, plasmid.

LS expression was first analyzed by RNA blotting of total RNA from pooled leaves (4th–10th verticils) of the nine T_0 independent transgenic lines. All lines but one (LS5) showed detectable levels of *LS* mRNA; the higher expressions were found in lines LS3, LS6, LS7 and LS9, whereas the rest of the lines showed a moderate expression of the transgene; no signal was detected in leaves from control plants (Fig. 2b).

RNA blotting of total RNA in flowers was performed in the six T_0 lines that flowered after 2 years growing in the greenhouse. Although all lines showed *LS* expression, lines LS3, LS4 and LS9 presented the highest levels (Fig. 2b). No signal was detected in flowers from control plants (Fig. 2c).

The expression of *LS* transgene was also determined in cohorts of leaves sampled at different developmental stages (I–V; Fig. 3a–c) of lines LS6 and LS7. In both lines, transgene expression was dependent on the developmental stage of the leaves, with the youngest (1st and 2nd verticils) showing the highest expression (Fig. 3d). The same expression pattern was found for the tubulin gene (Fig. 3e). No *LS* gene signal was detected in controls at any of the developmental leaf stages tested (Fig. 3d).

3.2. Molecular characterization of transgenic T_0 spike lavender progenies

The transgenic T_0 lines LS3 and LS8 produced seeds after self-pollination. The number of obtained T_1 plants was not enough to accurately analyze transgene inheritance. Nevertheless, Southern blotting patterns of the LS3-T_1 plants that inherited the *LS* transgene were similar to that of the two-copy parent (Fig. 4a), suggesting that both copies were integrated in the same chromosome. On the other hand, Southern blot of progenies from the four-copy parent LS8 displayed a variety of banding patterns corresponding to seven out of the twelve possible genotypes (Fig. 4d), which suggest that at least three of the four *LS* inserts were not linked.

To correlate phenotypes of LS-T_1 plants with gene expression, mRNA was determined for LS3 and LS8 progenies that did or did not inherit the *LS* gene. Total RNA was isolated from leaves of the 1st and 2nd verticils, and the expression of the transgenic *LS* cDNA was determined by Northern blot analysis. Only those progenies that inherited the transgene showed detectable levels of *LS* mRNA, and the expression was always higher in leaves from the first verticil (Fig. 4b, c, e and f). As shown in Fig. 5, Northern blotting of total RNA from leaves sampled at different positions in the stems of LS3-2, LS8-2 and LS8-5 plants, demonstrated that both *LS* and tubulin expression were also dependent on the developmental stage of the leaves, corroborating the results obtained with transgenic LS6 and LS7 T_0 lines (Fig. 3d and e).

3.3. Overexpression of the LS gene does not consistently affect essential oil profile in pooled leaves and flowers of transgenic T_0 spike lavender

The first series of essential oil analyses were accomplished in hydrodistillates of pooled air-dried leaves (verticils 4th–10th), consisting of both developing and mature leaves, and flowers from each transgenic T_0 and control plants. GC and GC/MS determinations allowed the identification of 28 constituents, accounting for 88.59–95.86% of the oils. In both transgenic and control plants monoterpenes were the main constituents of the oils (86.79–95.26% and 87.52–90.78% in leaves and flowers, respectively), followed by sesquiterpenes (0.11–3.06% and 0.91–3.68% in

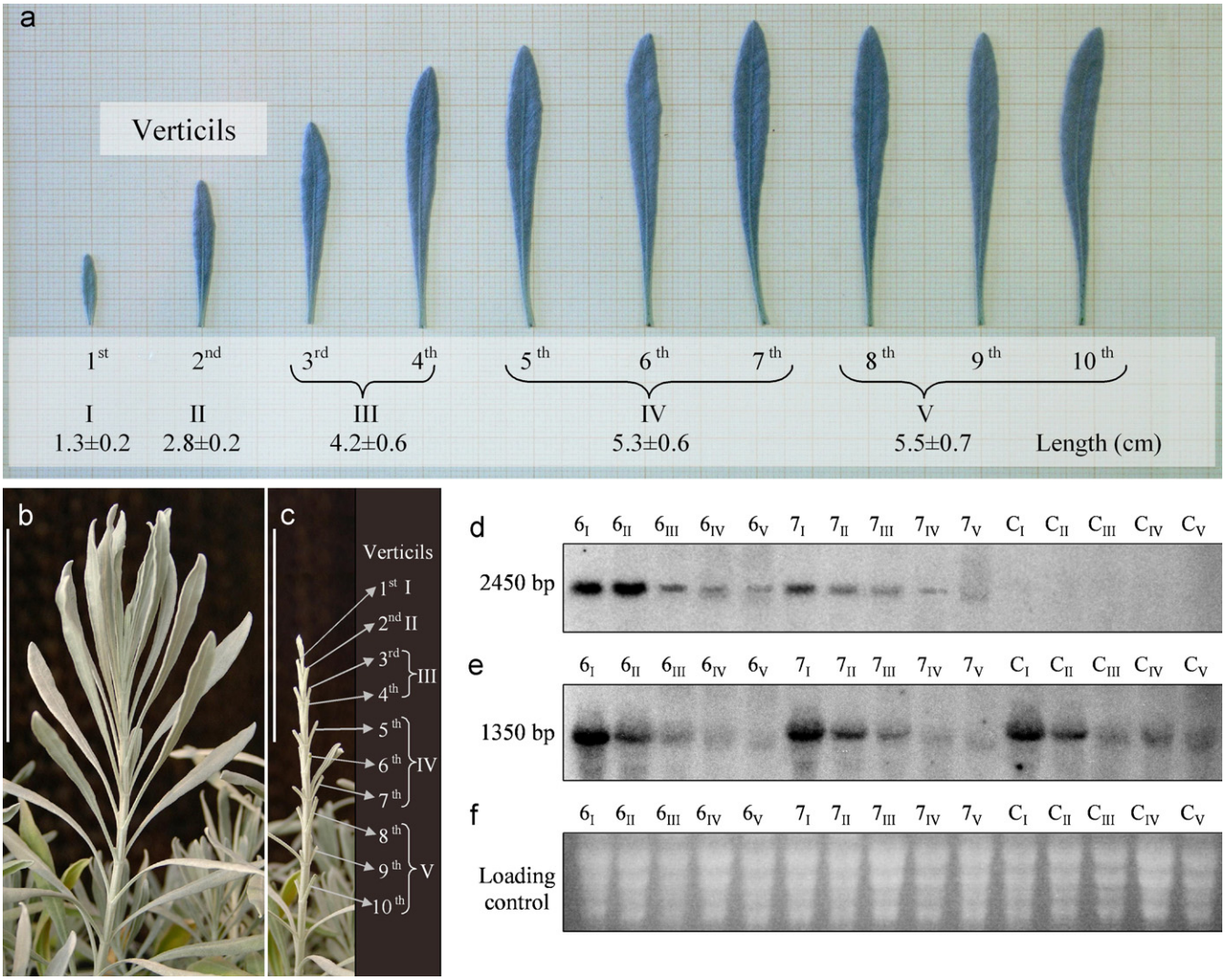

Fig. 3. Expression analysis of the *LS* gene during leaf development in transgenic T_0 spike lavender lines LS6 and LS7. (a) Sampled leaves from the 1st to 10th verticil grouped in five developmental stages (I–V). (b and c) Gross morphology of a vegetative stem (b; bar = 5 cm) showing the leaf sampling positions (c; bar = 5 cm). (d and e) RNA gel blot of *LS* (d) and tubulin 3 (e) transcript accumulation in leaves at five developmental stages (I–V). (f) Ribosomal RNA bands visualized with ethidium bromide and used to verify RNA loading before transfer. Lanes 6_I to 6_V, LS6; lanes 7_I to 7_V, LS7; lanes C_I to C_V, control.

leaves and flowers, respectively). The high percentage of monoterpenes was largely due to cineol and camphor in leaf oils (29.10–50.40% and 14.75–38.23%, respectively) and cineole, camphor and linalool in flower oils (10.06–34.85%, 17.00–34.00% and 14.53–43.35%, respectively).

To investigate whether *LS* overexpression affected the terpene profile of the spike lavender oil, we quantified along with limonene some of the most common produced acyclic (myrcene and linalool) and cyclic (α-pinene, cineole, camphor, α-terpineol and borneol) monoterpenes. The sesquiterpenes *t*-caryophyllene and its oxygenated derivative were also quantified. Most of these monoterpenes are direct products of the plastidial monoterpene synthases (Fig. 1), whereas the sesquiterpenes are synthesized in the cytosol. Because of this, if *LS* transgene is expressed, we expected that the quantities of some of these compounds would be modified due to changes in the precursor pool in both plastid and cytosol.

Limonene content in leaf oils of spike lavender increased more than 34.92% as compared to controls in three out of the eight transgenic lines that expressed the *LS* cDNA (lines LS2, LS4 and LS7; Table 1). Only one of these lines (LS4) also showed significant increases in α-pinene and myrcene content (27.91% and 21.82%, respectively), monoterpenes that can also be generated in small amounts by LS enzyme (Rajaonarivony et al., 1992). Changes in the amount of the other monoterpenes were dependent of the transgenic line and could not be clearly related to the variations of the three compounds directly formed by LS enzyme (Table 1). In flower oils (Table 2), three out of six T_0 lines that overexpressed the *LS* transgene increased their limonene production in relation to the controls more than 27.78% (lines LS2, LS3 and LS9). Furthermore, two of them (LS3 and LS9) also showed increased amounts of α-pinene (80.85% and 59.57%, respectively) and myrcene (18.18% and 29.09%, respectively). As in leaf oils, variations in the quantities of the other analyzed terpenes could not be related to the variations in limonene, α-pinene and myrcene (Table 2). Northern blot analyses (Fig. 2b and c) indicated that increases in the expression of the spearmint *LS* gene in either leaves or flowers did not correlate to limonene content (Tables 1 and 2).

3.4. Overexpression of the LS gene modifies essential oil profile in developing leaves of transgenic T_0 spike lavender and its progeny

To test the influence of the developmental stage of the leaf on essential oil composition, we analyzed extracts from cohorts of

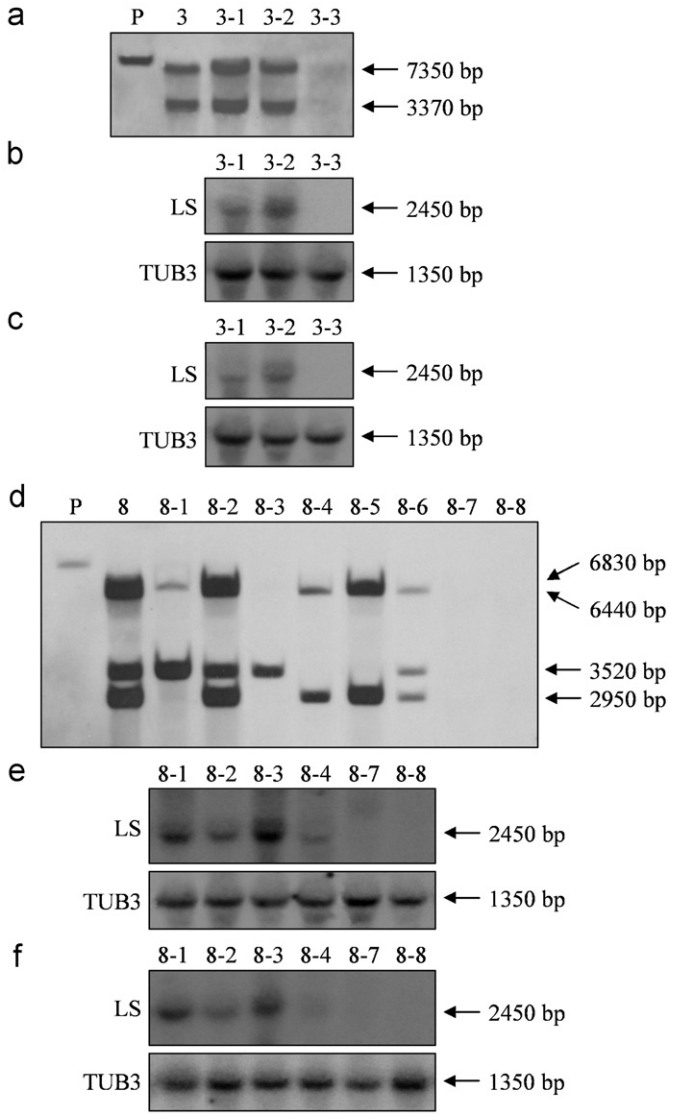

Fig. 4. Molecular analyses of transgenic T_1 spike lavender plants. (a and d) Southern blot hybridization of *LS* gene. (b, c, e and f) RNA gel blot of *LS* transcript accumulation in leaves of the 1st (b and e) and 2nd (c and f) verticil (developmental stages I and II; see Fig. 3a); the expression of the tubulin3 (*TUB3*) gene is shown to verify equal loading. Lane P, plasmid; lane 3, parental T_0 LS3; lanes 3-1 to 3-3, selected progenies of LS3 plant; lane 8, parental T_0 LS8; lanes 8-1 to 8-8, selected progenies of LS8 plant.

leaves sampled at different positions in the stems of LS6, LS7 and control plants (Fig. 3a–c). In both transgenic and control plants, the content of plastidial monoterpenes (limonene, α-pinene, myrcene, cineole, camphor, linalool, α-terpineol and borneol) was higher in the first verticil and decreased gradually with leaf development. Interestingly, the level of the cytosolic sesquiterpene *t*-caryophyllene followed the same pattern while its oxygenated derivative was undetectable in developing leaves, but its level increased gradually in older leaves (Table 3). The correlation between the amount of all the mono and sesquiterpenes tested and the leaf developmental stage was always significant (data not shown).

The effect of the transgene expression on oil composition was particularly striking in leaves from the first verticil (developmental stage I). In these leaves (Table 3), both LS6 and LS7 lines accumulated significantly more limonene, α-pinene and myrcene than the control plant (441.18%, 64.95% and 71.49% for LS6 and 514.67%, 102.21% and 92.66% for LS7, respectively). In contrast, levels of some of the monoterpene pathway components such as α-terpineol and cineole were significantly reduced compared to the control (−55.50% and −58.22% in LS6 and −52.11% and −62.46% in LS7, respectively). The rest of the analyzed monoterpenes and the two sesquiterpenes did not show consistent changes in their content compared to the control (Table 3). Note that although β-pinene was not quantified, its relative content (percentage in the leaf oil from the first verticil) also increased compared to the control (3.00% versus 1.70%, respectively).

For the rest of the verticils, the effect of the *LS* expression on oil composition decreased with leaf age as compared to the control (Table 3). Note however that for most of the leaf developmental stages, limonene content was always significantly higher in transgenic LS6 and LS7 lines than in the control (Table 3), corroborating results obtained when pooled leaves (4th–10th verticils) of the same transgenic lines were analyzed (Table 1). Furthermore, Northern blot analyses at the different leaf developmental stages indicated that increases in the expression of the spearmint *LS* cDNA paralleled increases in the limonene content of transgenic spike lavender LS6 and LS7 lines (Fig. 3d; Table 3).

Results from essential oil analyses in developing leaves (1st and 2nd verticils) of LS3 and LS8 progenies (Table 4) were similar to those previously reported in transgenic T_0 lines (Table 3). Regardless of the parental line, T_1 plants that inherited the *LS* gene maintained their elevated limonene phenotype; average limonene increases in LS3 and LS8 progenies that inherited the transgene were 241.02% and 175.72%, for the first verticil, and 202.50% and 158.56%, for the second verticil, respectively, as compared to their T_1 counterparts without

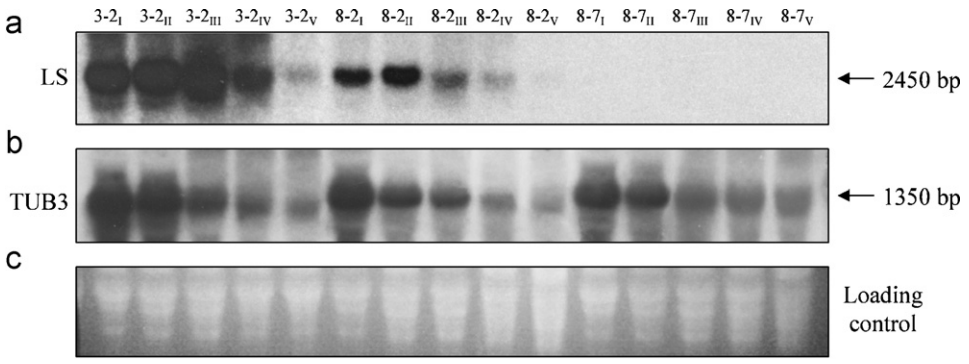

Fig. 5. Expression analysis of the *LS* gene during leaf development of selected transgenic T_1 spike lavender plants. (a and b) RNA gel blot of *LS* (a) and *TUB3* (b) transcript accumulation in leaves at five developmental stages (I–V; see Fig. 3a). (c) Ribosomal RNA bands visualized with ethidium bromide and used to verify RNA loading before transfer. Lanes 3-2_I to 3-2_V, progeny LS3-2 from parental T_0 LS3; lanes 8-2_I to 8-2_V and 8-7_I to 8-7_V, progenies LS8-2 and LS8-7, respectively, from parental T_0 LS8.

Table 1
Production (milligram per gram dried weight) of representative terpenes in leaf essential oils of control and transgenic T_0 spike lavender plants transformed with the spearmint *LS* gene[a]

Plants	Limonene	α-Pinene	Myrcene	Cineole	Camphor
Control	0.63±0.06 b[b]	1.29±0.31 bc	0.55±0.14 d	10.57±3.24 c	2.57±1.14 a
LS1	0.56±0.01 a	1.13±0.03 ab	0.33±0.01 a	7.32±0.06 ab	4.70±0.03 b
LS2	0.97±0.01 e	1.50±0.01 cd	0.46±0.00 bcd	14.22±0.03 de	11.14±0.05 e
LS3	0.60±0.01 ab	1.22±0.01 abc	0.34±0.00 ab	11.43±0.23 cd	5.20±0.05 b
LS4	0.85±0.00 d	1.65±0.02 d	0.67±0.00 e	16.09±0.07 e	5.27±0.02 b
LS5	0.78±0.02 c	1.60±0.02 d	0.55±0.01 cd	9.57±0.26 bc	5.38±0.03 b
LS6	0.75±0.01 c	1.25±0.01 abc	0.41±0.01 abc	6.25±0.04 a	7.19±0.05 c
LS7	0.91±0.03 d	1.60±0.05 d	0.58±0.03 de	8.11±0.13 ab	8.36±0.15 d
LS8	0.60±0.02 ab	1.04±0.01 a	0.35±0.00 ab	9.32±0.09 bc	4.88±0.02 b
LS9	0.58±0.02 ab	1.15±0.02 ab	0.54±0.00 cd	8.11±0.54 ab	2.07±0.00 a
Plants	Linalool	α-Terpineol	Borneol	*trans*-Caryophyllene	Caryophyllene oxide
Control	0.11±0.07 c	0.57±0.12 cd	0.09±0.09 a	0.10±0.10 b	0.08±0.04 b
LS1	0.03±0.01 a	0.46±0.03 ab	0.80±0.07 d	0.02±0.00 a	0.00±0.00 a
LS2	0.13±0.01 c	0.76±0.03 e	0.81±0.01 d	0.02±0.00 a	0.35±0.02 e
LS3	0.08±0.02 abc	0.53±0.03 bcd	0.31±0.05 c	0.09±0.00 ab	0.27±0.01 d
LS4	0.02±0.00 a	0.82±0.01 e	0.11±0.00 a	0.03±0.00 a	0.06±0.00 b
LS5	0.11±0.04 bc	0.64±0.01 d	0.22±0.00 b	0.03±0.00 a	0.69±0.00 f
LS6	0.07±0.00 abc	0.42±0.00 a	0.29±0.00 bc	0.02±0.00 a	0.26±0.10 d
LS7	0.09±0.00 abc	0.47±0.02 ab	0.28±0.01 bc	0.02±0.00 a	0.17±0.03 c
LS8	0.09±0.02 abc	0.53±0.00 abc	0.32±0.00 c	0.03±0.00 a	0.09±0.00 b
LS9	0.04±0.00 ab	0.50±0.01 abc	0.10±0.00 a	0.17±0.00 b	0.11±0.00 b

[a] Reported values for transgenic plants represent the means±SD of four measurements. The control value represents the average of four wild-type plants with four measurements each.
[b] Within each column, values followed by the same letter are not significantly different according to Tukey's test at $P \leqslant 0.05$.

Table 2
Production (milligram per gram dried weight) of representative terpenes in flower essential oils of control and transgenic T_0 spike lavender plants transformed with the spearmint *LS* gene[a]

Plants	Limonene	α-Pinene	Myrcene	Cineole	Camphor
Control	0.72±0.06 a[b]	0.94±0.22 a	0.55±0.10 a	8.78±1.91 b	8.09±2.15 bc
LS1	0.68±0.02 a	1.21±0.09 a	0.72±0.06 cd	10.80±0.91 c	7.05±0.27 b
LS2	1.32±0.06 e	2.28±0.17 d	0.51±0.05 a	7.40±0.40 ab	6.74±0.48 ab
LS3	0.92±0.01 c	1.70±0.04 c	0.65±0.04 bc	12.30±2.09 cd	8.25±0.66 bc
LS4	0.88±0.02 c	1.70±0.07 c	0.81±0.01 d	14.21±1.11 d	9.76±0.83 bc
LS8	0.81±0.02 b	1.08±0.04 ab	0.60±0.03 ab	6.87±0.41 a	14.84±1.12 e
LS9	1.18±0.03 d	1.50±0.09 c	0.71±0.03 cd	13.17±1.74 d	4.83±1.13 a
Plants	Linalool	α-Terpineol	Borneol	*trans*-Caryophyllene	Caryophyllene oxide
Control	11.51±0.84 d	0.38±0.23 b	0.81±0.29 a	0.56±0.30 b	0.03±0.01 b
LS1	9.91±0.25 c	0.66±0.06 de	0.98±0.02 ab	0.29±0.01 a	0.02±0.00 a
LS2	2.27±0.18 a	0.42±0.06 ac	1.29±0.08 c	0.85±0.04 c	0.03±0.00 b
LS3	8.77±0.02 c	0.55±0.09 bcd	1.38±0.06 c	0.81±0.16 c	0.04±0.01 b
LS4	22.90±0.46 f	0.82±0.01 e	0.87±0.07 a	0.47±0.01 ab	0.03±0.00 b
LS8	21.53±1.61 e	0.17±0.01 a	1.19±0.08 bc	0.34±0.02 ab	0.03±0.00 b
LS9	3.19±0.67 a	0.62±0.03 cde	1.34±0.19 c	0.46±0.01 ab	0.01±0.00 a

[a] Reported values for transgenic plants represent the means±SD of four measurements. The control value represents the average of four wild-type plants with four measurements each.
[b] Within each column, values followed by the same letter are not significantly different according to Tukey's test at $P \leqslant 0.05$.

the transgene (Table 4). The same behavior was observed in the content of the two other monoterpenes (α-pinene and myrcene) that also can be produced by the LS enzyme (Table 4). The *LS* transcript accumulation pattern was concordant with limonene, α-pinene and myrcene content; note that the LS8-4 plant that showed the lower level of *LS* mRNA also had the lower increase in these monoterpenes (Fig. 4b, c, e and f; Table 4).

We finally randomly chose three of the T_1 plants (LS3-2, LS8-2 and LS8-7) to analyze essential oil composition in leaves sampled at the five developmental stages. The results obtained showed that the temporal monoterpene accumulation pattern observed in leaves from the T_0 plants (Table 3) was stable in the subsequent generation; also the developmental expression of the *LS* transcript paralleled with limonene accumulation in leaves (Fig. 5a; Table 5). All these results demonstrated that *LS* transgene was stably transmitted to the progeny.

3.5. Overexpression of the LS does not alter the photosynthetic pigment content of spike lavender leaves

Metabolic engineering of monoterpenes might cause a reduced supply of precursors to branching (primary metabolites

Table 3
Effect of leaf developmental stage (LDS; see Fig. 3a) on the production (microgram per gram fresh weight) of representative terpenes in essential oil of transgenic T_0 spike lavender lines LS6, LS7 and control[a]

LDS	Plants	Limonene	α-Pinene	Myrcene	Cineole	Camphor
I	LS6	136.16±34.08 b[b]	202.96±54.45 b	92.50±25.30 b	2304.43±405.53 a	4288.78±586.86 a
	LS7	154.65±28.49 b	248.80±40.14 b	103.92±21.15 b	2070.55±264.85 a	4720.34±283.79 a
	Control	25.16±7.42 a	123.04±14.73 a	53.94±2.76 a	5516.25±562.18 b	4254.83±131.78 a
II	LS6	49.36±6.51 c	28.70±13.27 a	27.72±4.71 b	2046.32±29.05 a	3583.62±69.61 c
	LS7	34.56±1.13 b	17.08±2.03 a	18.12±0.34 a	1574.45±166.02 a	3151.25±347.49 b
	Control	24.64±4.52 a	28.03±10.47 a	22.33±3.56 ab	4478.71±951.72 b	2413.99±148.70 a
III	LS6	24.12±2.25 ab	9.14±1.72 a	12.72±1.53 ab	337.17±35.91 a	651.24±152.00 a
	LS7	31.41±9.30 b	10.95±2.87 a	16.51±4.68 b	276.22±119.28 a	511.23±245.57 a
	Control	15.61±0.53 a	9.20±1.48 a	9.74±0.36 a	632.54±403.24 a	312.1±168.70 a
IV	LS6	10.02±0.64 b	6.96±0.42 b	4.31±0.10 c	65.74±6.90 a	140.98±5.00 b
	LS7	11.44±0.84 c	6.61±0.92 b	3.73±0.28 b	68.02±1.80 a	161.44±9.62 c
	Control	5.04±0.43 a	4.12±0.36 a	3.16±0.28 a	109.81±10.89 b	68.41±6.12 a
V	LS6	9.01±1.89 b	6.87±0.42 b	2.08±0.28 b	57.90±15.05 a	172.15±49.97 b
	LS7	8.16±2.97 b	5.20±1.79 b	1.82±0.58 ab	64.93±14.23 a	181.83±22.13 b
	Control	3.47±0.97 a	2.73±0.45 a	1.29±0.17 a	105.12±35.77 b	90.56±21.98 a
LDS	Plants	Linalool	α-Terpineol	Borneol	*trans*-Caryophyllene	Caryophyllene oxide
I	LS6	6.39±1.01 a	258.25±47.64 a	320.69±50.35 a	8.13±1.68 a	0.00±0.00
	LS7	7.20±0.93 a	277.88±36.01 a	380.63±15.99 a	10.70±2.43 a	0.00±0.00
	Control	6.80±0.49 a	580.28±72.22 b	351.0±13.30 a	10.63±1.96 a	0.00±0.00
II	LS6	4.57±0.18 a	221.24±1.40 a	193.92±52.77 a	3.61±0.96 a	0.00±0.00
	LS7	3.75±0.60 a	179.50±25.78 a	174.67±58.86 a	5.14±2.68 a	0.00±0.00
	Control	4.34±0.90 a	448.14±103.32 b	202.61±9.62 a	3.85±1.10 a	0.00±0.00
III	LS6	1.01±0.14 a	56.75±2.06 a	17.36±2.88 b	2.14±1.03 a	0.00±0.00
	LS7	1.19±0.48 a	54.36±11.72 a	7.40±0.42 a	2.35±1.41 a	0.00±0.00
	Control	0.78±0.38 a	86.91±35.87 a	14.01±7.85 ab	1.35±1.12 a	0.00±0.00
IV	LS6	0.34±0.01 b	24.85±1.58 a	1.66±1.92 a	1.03±0.28 a	0.95±0.68 a
	LS7	0.38±0.03 c	24.20±0.66 a	2.07±2.40 a	1.13±0.16 a	1.81±0.06 a
	Control	0.24±0.02 a	28.73±5.31 a	1.40±1.62 a	1.32±0.20 a	0.58±0.40 a
V	LS6	0.35±0.15 a	20.48±0.57 a	4.95±1.47 b	0.61±0.32 a	3.64±1.23 a
	LS7	0.37±0.18 a	20.93±1.31 a	5.38±0.72 b	0.62±0.52 a	3.80±0.98 a
	Control	0.20±0.06 a	22.00±1.31 a	3.21±0.81 a	0.39±0.11 a	2.51±0.56 a

[a] Reported values for each T_0 plant represent the mean±SD of four measurements. The control value represents the average of four wild-type plants with four measurements each.
[b] Within each LDS and terpene, values followed by the same letter are not significantly different according to Tukey's test at $P \leqslant 0.05$.

pathways), thus imposing a cost on plant growth and fitness. Because of this, and to substantiate the visual morphological analyses of the plants, we quantified chlorophylls and carotenoids in spike lavender leaves from transgenic T_0 and controls. Total chlorophyll and carotenoid content depended on the analyzed plant (data not shown); nevertheless, the average amount (mg/g fresh weight) of the two photosynthetic pigments in transgenic and control plants was similar (1.76±0.21 versus 1.69±0.20 and 0.40±0.04 versus 0.39±0.05 for chlorophylls and carotenoids, respectively). None of the transgenic plants displayed visual phenotypes as compared to untransformed controls.

4. Discussion

Monoterpene synthases are involved in the regulation of pathway flux because they operate at a metabolic branch point and catalyze the first committed step leading to the different monoterpene families (Bohlmann et al., 1998). Thus, manipulating the expression of monoterpene synthase genes could be a tool for modifying aroma profiles of essential oil plants. This approach has been successfully employed for genetically imparting the ability to produce monoterpenes in a range of plant species and/or plant organs that do not naturally do so (Degenhardt et al., 2003; Aharoni et al., 2005; Wu et al., 2006). To date, however, these studies are scarce in aromatic species like spike lavender, with specialized secretory structures for trafficking and storage of these metabolites. Because of this, we have used the spearmint *LS* gene under the control of the constitutive CaMV 35S promoter to test whether up-regulation of LS could alter oil composition in spike lavender.

As in other lamiaceae species (Gershenzon et al., 2000), the loss of monoterpenes by catabolism and volatilization in spike lavender probably occurs at very low rate; thus, our first thought was to consider that the metabolic consequences of the

Table 4
Production (microgram per gram fresh weight) of representative terpenes in leaf essential oil of the first and second verticil [leaf developmental stages (LDS) I and II; see Fig. 3a] from transgenic T_1 spike lavender plants obtained from controlled self-pollination of T_0 transgenic LS3 and LS8 lines[a]

Plants	LDS	Limonene	α-Pinene	Myrcene	Cineole	Camphor
LS3-1	I	188.40±6.06 c[b]	337.30±41.41 c	122.69±13.50 c	3037.18±236.27 cd	5360.01±186.06 d
	II	71.99±4.98 b	63.53±6.03 b	45.90±4.94 b	1998.29±353.24 b	2776.26±333.74 b
LS3-2	I	183.45±7.33 c	305.76±60.25 c	122.48±19.58 c	3525.61±341.05 de	5267.40±259.87 d
	II	77.02±25.41 b	72.56±29.04 b	55.64±19.76 b	2409.58±363.55 bc	2326.10±402.39 b
LS3-3[c]	I	54.52±4.41 b	97.04±8.22 b	35.53±3.10 ab	3805.14±312.05 e	3621.85±297.34 c
	II	24.63±4.91 a	9.24±1.88 a	15.51±2.98 a	1267.34±266.56 a	1212.59±256.63 a
LS8-1	I	132.93±2.49 f	162.84±3.46 e	114.33±0.50 f	3797.99±93.43 cde	4944.61±199.30 e
	II	53.51±7.44 cd	27.53±7.81 abc	40.99±5.28 cd	1811.75±235.91 a	2733.20±301.42 bc
LS8-2	I	197.58±13.44 h	317.90±15.85 g	132.37±8.30 g	3058.17±421.69 bc	5287.17±102.17 e
	II	66.67±15.16 de	43.23±18.55 cd	38.56±9.82 cd	1919.62±395.34 a	3168.97±464.68 c
LS8-3	I	157.51±6.79 g	231.94±8.59 f	128.84±3.55 fg	3367.48±152.82 bcd	4806.51±72.56 de
	II	58.07±10.50 cd	34.69±12.53 bc	42.15±7.64 d	1788.39±290.57 a	2753.08±348.34 bc
LS8-4	I	80.24±4.36 e	58.25±3.43 d	97.05±5.29 e	4315.33±241.22 e	4518.65±249.59 d
	II	40.08±2.17 bc	16.18±0.89 ab	40.26±2.18 cd	1783.96±98.85 a	2345.19±130.10 b
LS8-7[c]	I	46.89±4.69 bc	38.38±5.44 cd	28.95±4.07 bc	3942.01±454.56 de	4769.67±319.71 de
	II	14.28±1.42 a	7.92±1.53 a	7.65±0.53 a	2043.13±188.36 a	1168.91±64.19 a
LS8-8[c]	I	56.16±7.40 cd	27.70±4.73 abc	29.95±4.29 bcd	4113.99±529.34 de	2519.23±325.82 b
	II	27.94±1.64 ab	8.88±0.60 a	17.32±1.00 ab	2964.58±187.77 b	1481.04±104.63 a
Plants	LDS	Linalool	α-Terpineol	Borneol	*trans*-Caryophyllene	Caryophyllene oxide
LS3-1	I	8.90±0.85 bc	639.64±41.39 d	404.56±33.69 c	11.13±1.25 b	0.00±0.00 a
	II	4.47±0.47 a	325.87±21.71 bc	183.54±24.76 b	4.30±0.40 a	0.21±0.41 a
LS3-2	I	9.15±0.71 c	901.53±63.21 e	394.28±34.19 c	11.62±1.70 b	0.00±0.00 a
	II	4.39±0.39 a	427.98±86.74 c	164.17±11.30 ab	5.64±0.76 a	0.33±0.65 a
LS3-3[c]	I	7.47±0.64 b	306.88±24.18 b	217.56±17.67 b	5.88±0.51 a	0.00±0.00 a
	II	4.32±0.86 a	210.24±39.56 a	124.78±25.57 a	3.77±0.76 a	0.00±0.00 a
LS8-1	I	11.56±0.10 e	469.23±15.53 fg	337.40±2.26 de	14.07±0.14 f	0.00±0.00 a
	II	6.42±0.76 abc	310.44±34.89 cd	178.34±19.02 c	5.74±0.51 bc	0.07±0.13 a
LS8-2	I	9.21±1.04 cde	351.59±26.57 de	406.33±32.48 e	11.89±0.93 e	0.00±0.00 a
	II	4.72±1.28 ab	213.37±43.43 b	196.71±34.74 c	3.46±0.65 a	0.00±0.00 a
LS8-3	I	10.43±0.44 de	424.41±2.24 ef	381.16±15.55 e	13.13±0.33 ef	0.00±0.00 a
	II	5.62±0.97 abc	272.55±38.79 bcd	193.53±26.70 c	4.76±0.58 ab	0.07±0.13 a
LS8-4	I	12.46±0.69 e	511.22±27.24 g	282.35±15.58 d	13.94±0.77 f	0.00±0.00 a
	II	7.19±0.39 abcd	350.76±18.40 de	160.05±8.83 c	6.54±0.36 cd	0.00±0.00 a
LS8-7[c]	I	12.54±4.34 e	447.75±73.57 fg	528.95±89.16 f	7.86±1.14 d	0.00±0.00 a
	II	4.17±0.14 a	113.36±0.69 a	155.62±3.89 bc	3.11±0.27 a	0.00±0.00 a
LS8-8[c]	I	7.81±0.99 bcd	332.99±40.66 d	79.62±10.30 ab	6.67±1.14 cd	0.27±0.00 b
	II	10.29±0.62 de	236.65±13.50 bc	55.45±3.80 a	4.31±0.26 ab	0.27±0.00 b

[a] Reported values for each T_1 plant represent the mean±SD of four measurements.
[b] Within each column and progeny (LS3 and LS8), values followed by the same letter are not significantly different according to Tukey's test at $P \leqslant 0.05$.
[c] T_1 plants that did not inherit the *LS* transgene.

constitutive expression of the *LS* gene in spike lavender could be accurately studied in pooled leaves (from 4th to 10th verticils) or flowers. Since monoterpene biosynthesis in some lamiaceae species is developmentally regulated (McConkey et al., 2000), this sampling method, also used in previous studies with transgenic spike lavender overexpressing the 1-deoxy-D-xylulose-5-phosphate synthase (*DXS*) or 3-hydroxy-3-methylglutaryl CoA reductase genes (Muñoz-Bertomeu et al., 2006, 2007b), was

Table 5
Effect of leaf developmental stage (LDS; see Fig. 3a) in the production (microgram per gram fresh weight) of representative terpenes of transgenic T_1 spike lavender LS3-2, LS8-2 and LS8-7[a]

LDS	Plants	Limonene	α-Pinene	Myrcene	Cineole	Camphor
I	LS3-2	183.45±7.33 b[b]	305.76±60.25 b	122.48±19.58 b	3525.61±341.05 ab	5267.40±259.87 b
	LS8-2	197.58±13.44 b	317.90±15.85 b	132.37±8.30 b	3058.17±421.69 a	5287.17±102.17 b
	LS8-7[c]	46.89±4.69 a	38.38±5.44 a	28.95±4.07 a	3942.01±454.56 b	4769.67±319.71 a
II	LS3-2	77.02±25.41 b	72.56±29.04 b	55.64±19.76 b	2409.58±363.55 a	2326.10±402.39 b
	LS8-2	66.67±15.16 b	43.23±18.55 b	38.56±9.82 b	1919.62±395.34 a	3168.97±464.68 c
	LS8-7[c]	14.28±1.42 a	7.92±1.53 a	7.65±0.53 a	2043.13±188.36 a	1168.91±64.19 a
III	LS3-2	45.51±17.42 b	23.53±18.62 b	18.83±12.28 b	1316.83±765.13 b	569.05±331.51 b
	LS8-2	30.25±1.88 b	6.53±1.15 ab	14.67±1.06 b	308.99±95.89 a	463.50±140.01 b
	LS8-7[c]	3.20±0.09 a	1.88±0.33 a	1.88±0.19 a	326.09±39.95 a	80.28±25.02 a
IV	LS3-2	12.68±8.33 b	6.11±3.10 b	3.29±2.04 b	493.83±321.27 b	205.73±131.65 b
	LS8-2	10.61±1.51 b	2.71±0.35 a	4.65±0.98 b	93.61±9.4 a	138.24±6.08 b
	LS8-7[c]	1.72±0.56 a	1.04±0.40 a	0.98±0.07 a	200.77±4.53 ab	12.62±4.39 a
V	LS3-2	6.70±2.78 b	3.49±1.52 a	1.53±0.52 a	359.02±210.53 b	163.48±78.25 b
	LS8-2	6.07±0.04 b	2.89±0.18 a	2.57±0.02 b	89.54±2.82 a	138.41±3.57 b
	LS8-7[c]	1.09±0.77 a	1.68±0.89 a	1.11±0.12 a	109.43±4.90 a	13.38±9.70 a
LDS	Plants	Linalool	α-Terpineol	Borneol	*trans*-Caryophyllene	caryophyllene oxide
I	LS3-2	9.15±0.71 a	901.53±63.21 c	394.28±34.19 a	11.62±1.70 b	0.00±0.00
	LS8-2	9.21±1.04 a	351.59±26.57 a	406.33±32.48 a	11.89±0.93 b	0.00±0.00
	LS8-7[c]	12.54±4.34 a	447.75±73.57 b	528.95±89.16 b	7.86±1.14 a	0.00±0.00
II	LS3-2	4.39±0.39 a	427.98±86.74 c	164.17±11.30 a	5.64±0.76 b	0.33±0.65 a
	LS8-2	4.72±1.28 a	213.37±43.43 b	196.71±34.74 a	3.46±0.65 a	0.00±0.00 a
	LS8-7[c]	4.17±0.14 a	113.36±0.69 a	155.62±3.89 a	3.11±0.27 a	0.00±0.00 a
III	LS3-2	2.17±1.35 b	148.85±72.34 b	27.02±13.81 b	1.12±0.20 a	0.83±0.96 a
	LS8-2	1.01±0.11 ab	57.75±7.69 a	13.40±5.87 ab	0.83±0.24 a	0.00±0.00 a
	LS8-7[c]	0.42±0.11 a	28.41±0.90 a	9.18±3.55 a	0.81±0.04 a	0.00±0.00 a
IV	LS3-2	0.66±0.38 b	56.03±25.28 b	12.74±6.57 b	0.12±0.12 a	1.24±0.26 b
	LS8-2	0.44±0.06 ab	33.15±1.90 ab	3.11±0.39 a	0.43±0.12 b	0.27±0.00 a
	LS8-7[c]	0.11±0.08 a	20.16±0.24 a	2.57±0.82 a	0.38±0.17 b	0.33±0.38 a
V	LS3-2	0.46±0.23 b	32.88±9.63 b	8.68±2.55 b	0.13±0.10 a	1.32±0.09 b
	LS8-2	0.45±0.01 b	28.05±0.72 ab	3.71±0.03 a	0.46±0.08 b	0.48±0.25 a
	LS8-7[c]	0.08±0.09 a	19.93±0.03 a	2.39±0.62 a	0.32±0.09 b	0.39±0.26 a

[a] Reported values for each T_1 plant represent the mean±SD of four measurements.
[b] Within each LDS and terpene, values followed by the same letter are not significantly different according to Tukey's test at $P \leqslant 0.05$.
[c] T_1 plant that did not inherit the *LS* transgene.

chosen to avoid physiological fluctuations independent of the presence of the transgene.

Some of the transgenic T_0 spike lavender lines overexpressing *MsLS* showed quantitative and qualitative alterations in their terpene profiles, particularly increased amounts of limonene as compared to controls (from 19.05% to 53.97% and from 12.50% to 83.33% in pooled leaves and flowers, respectively). However, the levels of other monoterpene pathway components were not consistently altered in either leaf or flower oils. Also, a positive correlation between limonene content and *LS* transgene expression in leaves or flowers could not be found. Previous work in mint species overexpressing *MsLS* also showed that monoterpene profile in pooled leaf samples was not clearly altered (Krasnyanski et al., 1999; Diemer et al., 2001). An insufficient amount of the LS protein in the glandular trichome sites of essential oil biosynthesis has been suggested as the main cause of the lack of effects on oil yield or composition in transgenic mint plants (Mahmoud et al., 2004).

It is well established that the developmental status of the leaves impacts the production of monoterpenes in several aromatic species (Dudai et al., 1999, 2001; Rohlof, 1999; Gershenzon et al., 2000; Turner et al., 2000). Nevertheless, it has not yet been shown that this developmental regulation could affect monoterpene production in transgenic aromatic plants overexpressing monoterpene synthase genes under the regulation of a constitutive promoter. Because of this, we analyzed leaf essential oil composition in leaves sampled at five different developmental stages in transgenic T_0 and control plants. The contents of the most common monoterpenes (limonene, α-pinene, myrcene, cineole, camphor, linalool, α-terpineol and borneol) found in spike lavender oils were higher in developing leaves from the first verticil and decreased over the organ's lifespan. The content of the sesquiterpene *t*-caryophyllene followed the same pattern, but that of its oxygenated derivative increased with leaf age. This strict developmental regulation of mono and sesquiterpene biosynthesis in spike lavender was reproducibly observed in transgenic progenies that did or did not inherit the spearmint *LS*.

Although LS transgene was driven by a CaMV 35S promoter, which implies expression in every leaf through development,

transcript abundance of *LS* gene was the highest in the youngest leaves. This was followed by a progressive decrease in RNA to reach a stable expression in fully developed leaves. Interestingly, the high *LS* expression in leaves from the first verticil correlated well with the highest limonene accumulation (average increase for T_0 lines LS6 and LS7 was 477.93% with respect to control). Both *LS* mRNA transcript abundance and limonene levels decreased with leaf age, but limonene content in most of the developmental leaf stages was significantly higher in transgenic plants as compared to the control. As the mRNA levels from the *LS* transgene closely tracked those from tubulin, a transcriptome-wide decrease in mRNA synthesis as leaves age is suggested; thus, monoterpene synthesis would be a function of the overall metabolic activity of the cells. Nevertheless, a clear correlation between *LS* expression and limonene content could not be established due mainly to a sharp descent in limonene content from the second verticil; thus, these results could also suggest that limonene synthesis in spike lavender glandular trichomes is regulated at both transcriptional and posttranscriptional (translational and/or posttranslational) levels as previously demonstrated in mint species (Gershenzon et al., 2000; McConkey et al., 2000). Besides this, precursor availability could also limit the production of limonene as already suggested for other monoterpenes (Dudareva et al., 2004). In fact, in a previous work, we found that overexpressing DXS, which catalyzes the first step of the MEP pathway, lead to a generalized increase in monoterpene content in transgenic spike lavender plants (Muñoz-Bertomeu et al., 2006). Similar results were obtained in transgenic peppermint overexpressing 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) that catalyzes de second step of the same pathway (Mahmoud and Croteau, 2001). Furthermore, in this species both transcript abundance of DXS gene and monoterpene production were highest in developing leaf oil glands, but little, if any, DXS expression and monoterpene production were observed in expanded mature leaves (Lange et al., 1998; Turner et al., 2000).

Since most monoterpene synthases utilize GPP as universal substrate, overexpressing one of them may cause alterations in the terpene profile of the essential oil. The study of such competition for the same limiting substrate is complicated in species like spike lavender, not only because they produce a high amount and diversity of terpenes, but also by the ability of several monoterpene synthases to produce multiple products (Bohlmann et al., 1998; Dudareva et al., 2006). For example, LS from spearmint transforms GPP into limonene, pinenes and myrcene (Colby et al., 1993), whereas 1,8-cineole synthase from *Salvia officinalis* can generate α-terpineol and cineole (Croteau et al., 1994). Altered levels of several monoterpene pathway components were reproducibly observed in both transgenic T_0 spike lavender plants and those progenies that inherited the *LS* gene. These changes were restricted to youngest leaves (first verticil) and lead to increases of pinenes and myrcene, the other compounds simultaneously produced by LS enzyme. In contrast, cineole and α-terpineol were significantly reduced, indicating a clear competition between 1,8-cineole synthase with LS for GPP.

With few exceptions, the MEP pathway provides IPP for both plastidial monoterpene and cytosolic sesquiterpene biosynthesis (reviewed by Eisenreich et al., 2004; Bouvier et al., 2005). Because of this, any change in the plastid precursor pool might affect sesquiterpene formation. Thus, the level of the sesquiterpene β-caryophyllene emitted by flowers of transgenic tobacco plants overexpressing three plastidil-targeted lemon monoterpene synthases was lower than the level in wild-type flowers (Lücker et al., 2004). In contrast to these results, the overexpression of *LS* gene did not alter sesquiterpene content in spike lavender.

In conclusion, this study demonstrates that monoterpene profile in spike lavender oils can be modified by overexpressing the *LS* gene from spearmint. The observed changes in essential oil composition of the transgenic plants are developmentally regulated, being especially evident in the youngest leaves. Significant oil profile changes in developed leaves are probably limited by GPP precursor availability. Based on our findings, it would be predicted that the co-expression of genes encoding regulatory enzymes of the MEP pathway, such as *DXS*, with monoterpene synthase genes would maximize the levels of particular monoterpenes in spike lavender oil; these approaches would be of interest to produce spike lavender plants of increased value. Besides this biotechnologically relevant item, plants overexpressing LS provide a valuable model for a better understanding of the monoterpene synthesis and regulation in aromatic plants.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2008.04.002.

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