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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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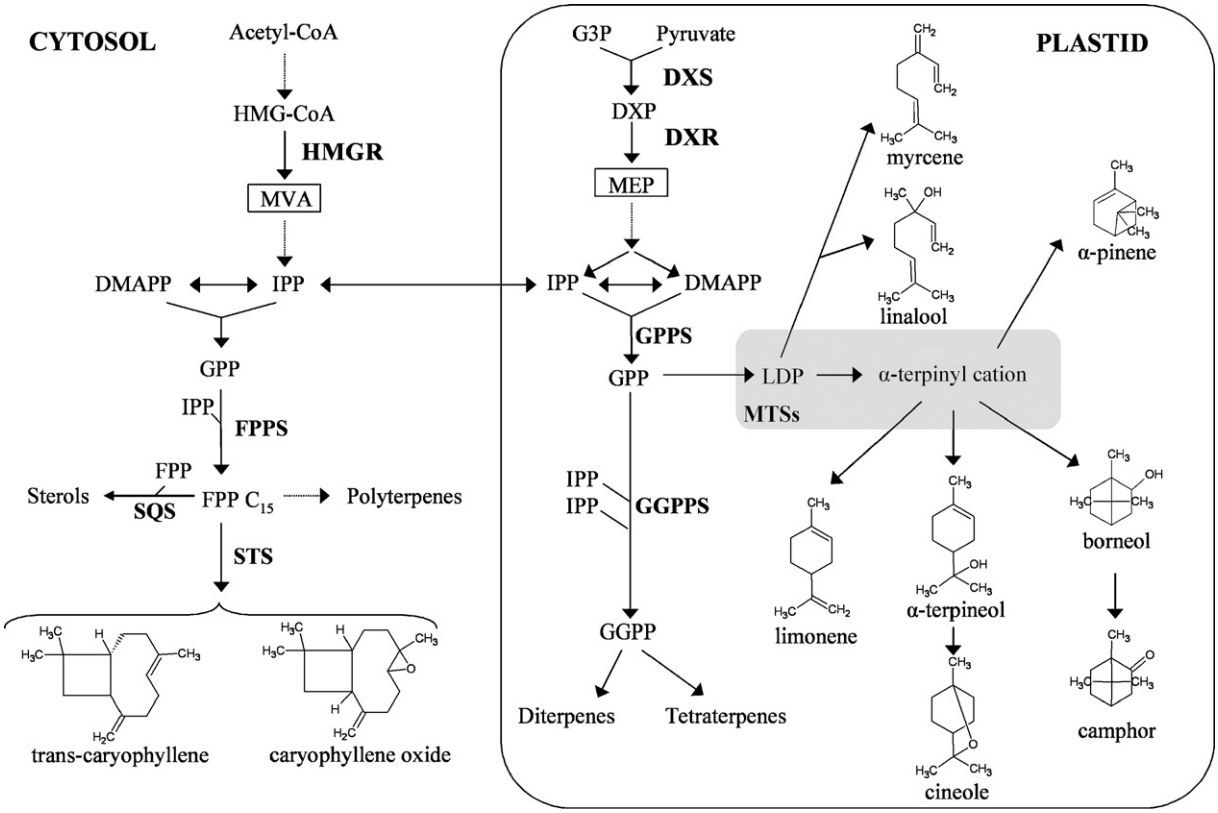

Fig. 1. Isoprenoid biosynthesis in plants. Enzymes are indicated in boldface: DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; FPPS, farnesyl diphosphate synthase; GGPPS, geranylgeranyl diphosphate synthase; GPPS, geranyl diphosphate synthase; HMGR, 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),