

Monoterpene production and regulation in
Lavenders
(*Lavandula angustifolia*
and
Lavandula x intermedia)

by

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ABSTRACT

Lavenders (*Lavandula*) are widely grown for their essential oils, which have extensive applications in cosmetics, hygiene products and alternative medicine. The therapeutic and olfactory properties of lavender essential oils are attributed to monoterpenes, a class of low molecular weight (C₁₀) isoprenoids. Oil composition in these plants is primarily determined by plant genotype, but can also be influenced by developmental and environmental factors.

In order to define some of the mechanisms that control monoterpene abundance in lavenders, I measured the abundance of quality-defining monoterpenes in several *L. angustifolia* and *L. x intermedia* cultivars grown in the Okanagan. Data obtained confirmed that essential oil yield, as well as the abundance of camphor, borneol, linalool, and limonene was species-specific. *L. angustifolia* cultivars contained high amounts of linalool but yielded little oil, whereas *L. x intermedia* cultivars were rich in camphor and total oil. Monoterpene abundance changed during flower development, and differed between vegetative and reproductive tissues indicating differential regulation of the biosynthetic pathways, or specialized ecological functions. The abundance of linalool correlated with the transcription of the linalool synthase gene, suggesting that linalool production is in part regulated transcriptionally. However, the degree of correlation between linalool abundance and linalool synthase transcription differed between *L. angustifolia* and *L. x intermedia*, suggesting additional, and differing mechanisms that control linalool abundance in these species. In addition, monoterpene abundances were subject to loss during storage and suboptimal detection, two factors that must be considered in future analyses. Results obtained in this study provide insight into the regulation of monoterpene production in lavenders, and build the basis for future research aimed at improving essential oil production in these plants.

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LIST OF ABBREVIATIONS

Aa	Amino acid
BDH	Bornyl dehydrogenase
Bp	Base pair
BPP	Bornyl diphosphate
BPPH	Bornyl diphosphate hydrolase
BPPS	Bornyl diphosphate synthase
carb ⁵⁰	50ug/ml carbenicillin
cDNA	Complementary DNA
cv.	Cultivar
DMAPP	Dimethyl-allyl diphosphate
DXP	Deoxy-xylulose-phosphate
EI mode	Electro ionization mode
GC/MS	Tandem Gas chromatography mass spectrometry
GPP	Geranyl diphosphate
IPP	Isopentyl diphosphate
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ISO	International organization for standardization
LIS	Linalool synthase
<i>Lxi</i> -LIS	<i>Lavandula x intermedia</i> LIS
MEP	Methyl-erythriol phosphate
mg/gfWT	mg per gram fresh weight
mRNA	Messenger RNA
MTS	Monoterpene synthase
MVA	Mevalonic acid
n.d.	Non-detected
n/a	Not available
ORF	Open reading frame
PCR	Polymerase chain reaction
RT-PCR	Reverse-Transcriptase PCR
SDSE	Steam distillation solvent extraction
SDS-gel	Sodium dodecyl sulfate gel
SE	Standard error
TPS	Terpenoid synthase
U	Unit- amount of the enzyme that catalyzes the conversion of 1 micro mole of substrate per minute

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1. INTRODUCTION

Lavender has been known and cultivated for centuries. The first written accounts can be traced back to Dioscorides in about AD 65 (Upson and Andrews, 2004), while reports on its application go as far back as to Egyptian times where lavender flowers were used in the mummification process (Chu and Kemper, 2001). The name of the genus *Lavandula* and its common name lavender is generally believed to be derived from Latin, *lavare*, to wash. However, since no written evidence exists to corroborate the use of lavender products in Roman or Greek bathing, it is rather likely that *Lavandula* and lavender arose from the Latin '*livere*', 'to be livid or bluish' in reference to the flower colour (Upson and Andrews, 2004).

1.1. The genus *Lavandula*

Lavandula, along with representatives of other genera like common sage (*Salvia sp.*), mint (*Mentha sp.*) and thyme (*Thymus sp.*), is a member of the *Lamiaceae* family. The genus consists of 25-35 sub-species, which display a diverse morphology. Lavenders are defined and distinguished from all other *Lamiaceae* by the morphology of their flower. Their characteristic compact terminal flower spike is borne on a long peduncle (flower stalk). The flower spike consists of cymes, a branching determinate inflorescence with a flower at the end of each branch, either in an opposite decussate or alternate spiral arrangement, which are subtended by bracts (Lis-Balchin, 2002). In habit, lavenders vary from woody shrubs up to a meter in height, to perennial woody-based shrubs or annual herbs. The leaves can be entirely or deeply dissected and are often absent in some Arabian species (Lis-Balchin, 2002).

According to morphological characteristics, the genus *Lavandula* is subdivided into three subgenera: *Lavandula*, *Fabricia* and *Sabaudia*. Each subgenus is further diversified into sections, which dissociate into numerous species. For example, *Lavandula* encompasses the sections *Lavandula*, *Dentatae* and *Stoechas*; *L. angustifolia*, *L. latifolia* and *L. lanata* represent different species and Munstead is a *L. angustifolia* cultivar (Figure 1). It is important to note that there are also intra-and intersectional hybrids, which arise from the natural or artificial crossing of two different species. Lavandin (*Lavandula x intermedia*) is derived from a cross of *L. latifolia* x *L. angustifolia*. It is extensively cultivated and one of the most prominent

lavenders worldwide (Upson and Andrews, 2004). Grosso is a well known representative of this hybrid species (for complete reviews on lavender taxonomy refer to Lis-Balchin, 2002 and Upson and Andrews, 2004).

1.2. Lavender: Medicinal and commercial application

Lavender has a long-standing history as a medical remedy. Lavender extracts have traditionally been prescribed to treat infertility, infection, anxiety and fever, and have been used as antidepressants, antispasmodics, antifatulent agents, antiemetic remedies and diuretics (Chu and Kemper, 2001). In recent years, lavender essential oil has gained a strong reputation in aromatherapy and as a holistic relaxant to treat stress, anxiety, depression, fatigue or insomnia (Chu and Kemper, 2001). Studies suggest that lavender aroma during recesses prevents deterioration of work performance (Sakamoto et al., 2005) and might improve memory and cognition in Alzheimer's patients (Adseren et al., 2005). There has been increasing interest in perillyl alcohol, a monoterpene found in trace amounts in *L. angustifolia* (Perrucci et al., 1994), due to its chemopreventative and chemotherapeutic properties (Schulz et al., 1994, Hohl, 1996, Peffley and Gayen, 2003).

Next to its use as medicine, lavender is valued for its strong and pleasant fragrance. In Victorian times lavender was known as an aphrodisiac; in the Medieval and Renaissance periods it was used for the storage of laundry and to disguise objectionable odours (Chu and Kemper, 2001). Nowadays, we commonly find lavender in a wide variety of perfumes and soaps with the lavender-based perfumery/cosmetic industry growing worldwide.

1.3. Lavender essential oil

The medicinal and olfactory properties of lavender oil are mainly attributed to monoterpenes, a class of volatile organic compounds that constitute lavender essential oil and give lavender its characteristic aroma. Forty to fifty different monoterpenes can generally be identified in lavender essential oil, with linalool, linalool acetate, 1,8-cineol, β -ocimene (usually both *cis*- and *trans*-), terpinene-4-ol and camphor as the major constituents (Kreis and Mosandl, 1992, Flores et al., 2005).

The proportional composition of these compounds determines the quality of the essential oil. High quality oil used in perfumery generally contains high percentages of linalool and linalool acetate, while the oil fragrance deteriorates with increasing camphor ratios (Adam 2004). The quality of medicinally-utilized oil on the other hand is determined by the proportion of monoterpenes with the desired biological activity. For instance, linalool acetate and linalool, found in high amounts in *L. angustifolia*, have sedative, and local anesthetic effects. 1,8-cineole, comprising over 50% of the essential oil of *L. dentate*, acts as a spasmolytic, local anesthetic and antibacterial agent. Camphor, found in high concentrations in *L. latifolia*, α -terpineol and terpenen-4-ol have antibacterial properties. Alpha-pinene, 1,8-cineole, β -pinene and p-cymene have antifungal activity, while caryophyllene oxide, a sesquiterpene found in *L. latifolia* and *L. angustifolia*, has anti-inflammatory effects (Chu and Kemper, 2001). As exemplified for camphor proportions in lavender oil, oil classified as low quality for perfumery, might be regarded as high quality oil when used medicinally.

The composition of an essential oil greatly depends on the species it is derived from (Cavanagh and Wilkinson, 2002). As an example, the oil composition of three of the most common lavenders, *Lavandula angustifolia* (formerly *L. officinalis*, English lavender), *L. latifolia* (Spike lavender) and *L. x intermedia* (Lavandin) is given in Table 1.

Some of the finest oils are extracted from *L. angustifolia*, which has the highest ratio of linalool to camphor. However, this species is small and difficult to propagate and hence low in overall oil yield. Lavandin displays a less favourable linalool to camphor composition, but plants are hardier and produce greater amounts of oil per acre even in cold climates like the UK (Interactive European Network for Industrial Crops and their Applications (IENICA) September 27, 2002). The choice of lavender variety is therefore a function of required oil yield, required quality (higher quality oils for pure essential oils, fragrances and medical application, lower quality oils for soaps and detergents) and the growth environment.

1.4. Terpene biosynthesis

Linalool and camphor are chemically characterized as monoterpenes. Monoterpenes, the main constituents of lavender essential oil, belong to a large and diverse group of chemical compounds termed ‘terpenes’. Terpenes are naturally occurring organic hydrocarbons, also known as isoprenoids or terpenoids after oxidation or rearrangement of the carbon skeleton.

All terpenes are derived from the condensation of the 5-carbon unit isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Depending on the number of isoprene units linked together, terpenes are classified by size into hemiterpenes (C₅), monoterpenes (C₁₀), sesqui- (C₁₅), di- (C₂₀), sester-, tri-, and tetraterpenes (C₂₅, C₃₀, C₄₀, respectively).

1.4.1. Isoprene biosynthesis

Until recently, it was assumed that IPP and DMAPP were exclusively synthesized from mevalonate in the cytosol via the so called mevalonate or MVA pathway. In the first step of this pathway, three molecules of acetyl-coenzyme (Co)A couple to yield 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), which is subsequently reduced by the enzyme HMG-CoA reductase (HMGR) to yield mevalonic acid (MVA). In the next two steps, mevalonate kinase and mevalonate 5-phosphate kinase phosphorylate MVA to form mevalonate 5-diphosphate, which is subsequently decarboxylated to yield IPP (Figure 2) (Liu et al., 2005). Flux through this pathway is regulated by the activity of HMGR in mammals and fungi (Chappell et al., 1995). Studies investigating the regulatory role of HMGR in plants generally produced controversial results. Overexpression of Hamster HMGR in tobacco plants for instance favoured the accumulation of total sterols, while levels of other isoprenoids such as carotenoids or the phytol chain of chlorophyll remained relatively unaltered in the transgenic plants (Chappell et al., 1995). This controversy was later rationalized by the discovery of a novel mevalonate independent pathway for IPP synthesis in plants (Rodriguez-Concepcion and Boronat, 2002) and bacteria (Rohmer et al., 1993). This new pathway, commonly referred to as DXP or MEP pathway, begins with a transketolase type condensation of pyruvate and glyceraldehyde-3-phosphate to 1-deoxy-D-xylulose 5-phosphate (DOXP), catalyzed by DOXP synthase (DXPS). DOXP is reduced by DX reductoisomerase (DXR) to 2-C-Methyl-D-erythritol 4-phosphate (MEP). MEP synthesis is followed by formation of the cytidine 5-diphosphate derivative, phosphorylation and cyclization to 2- C-methylethritol-2,4-cyclodiphosphate (MECP). MECP is then converted to 1-hydroxy-2 methyl-2-(E)- butenyl 4-diphosphate (HMBPP) by HMBPP synthase. Isopentenyl diphosphate and DMAPP are produced as final products (Figure 2) (Liu et al., 2005). In higher plants, this pathway is localized to plastids where it is the main source for precursors of hemiterpenes, monoterpenes,

diterpenes and carotenoids. Precursors for sterols, sesquiterpenes and ubiquinones on the other hand, are mainly derived from the mevalonate route operating in the cytoplasm and mitochondria (Lichtenthaler, 1999).

Transgenic manipulation of the DXP pathway in *E. coli* and plants suggested that metabolic flux through this pathway is regulated by DXPS, DXR and HMBPP reductase. Overexpression or suppression of DXPS in *Arabidopsis* and tomato led to the respective increase or decrease in isoprenoid production (Estevez et al., 2001, Rodriguez-Concepcion et al., 2001). Hence, DXPS is one of the limiting steps in the production of plastidal IPP. Similarly, ectopic expression of DXR in peppermint caused an increase in the production of monoterpenes by 40%-60% (Mahmoud and Croteau, 2001). Finally, overexpression of HMBPP reductase, which catalyzes the simultaneous synthesis of IPP and DMAPP in the last step of the MEP pathway, triggered increased production of isoprenoids in tomato and *Arabidopsis* (Botella-Pavia et al., 2004).

Information on the regulation of the crosstalk between the MVA- and DXP pathways is sparse (Bouvier et al., 2005, Eisenreich et al., 2004, Rodriguez-Concepcion et al., 2004). However, it has been shown that exchange of metabolites between these two pathways is possible (Bick and Lange, 2003, Laule et al., 2003, Schuhr et al., 2003, Dudareva et al., 2005, Hampel et al., 2005, Hemmerlin et al., Cusidó et al., 2007). McCaskill et al. (1995) and Laule et al. (2003) demonstrated that intermediates generated in the DXP pathway compensate for reduced flux through the mevalonate pathway (McCaskill and Croteau, 1995, Laule et al., 2003), and overexpression of HMGR in spike lavender increased the abundance of sterols as well as mono- and sesquiterpenes. The abundance of carotenoids or chlorophylls, however, remained unaffected (Munoz-Bertomeu et al., 2007). Thus, monoterpenes (as well as hemiterpenes, diterpenes and carotenoids) are not necessarily or exclusively produced through the DXP pathway. However, the nature of this metabolite exchange and its regulation has yet to be established.

1.4.2. Biosynthesis of higher terpenes (condensation of IPP and DMAPP)

In the second part of terpene synthesis, IPP and DMAPP, derived from either the MVA- or DXP pathway, are condensed to yield geranyl diphosphate (GPP), farnesyl diphosphate (FPP) or geranylgeranyl diphosphate (GGPP). The initial step involves the

isomeration of IPP to DMAPP by IPP isomerase. DMAPP is the more reactive compound, i.e. its allylic phosphate group is an excellent leaving group that yields a carbonium ion. Through the catalytic action of GPP synthase (a prenyltransferase), the carbonium ion then acts as an alkylating agent in the reaction with IPP, giving GPP. In plastids, GPP is converted either to monoterpenes or utilized for the production of GGPP via three condensation steps by GGPP synthase and the addition of two IPP molecules to one molecule of GPP (Figure 3). In the cytosol, GPP is utilized to produce FPP through the condensation of one molecule of GPP with one molecule of IPP by FPP synthase. FPP is the precursor for sesquiterpenes (Figure 3) in the cytosol. Higher terpenes are synthesized by a continuation of this chain extension process, either through the addition of IPP to GGPP to yield sesterterpenes (C₂₅) (in plastids) or through condensation of two FPP, or two GGPP to generate triterpenes (C₃₀) in the cytosol, or to generate tetraterpenes (C₄₀) in plastids (for review see Liu et al., 2005).

A wide array of terpenoids found in essential oils, turpentine and plant resins are produced by the cyclization of GPP, FPP or GGPP, and subsequent transformation of these parent skeletons by a series of redox isomerization and conjugation reactions. The plethora of different monoterpenes results from the derivatization of GPP and the rearrangement of the primary monoterpene skeletons (Figure 4). Since monoterpenes represent the major constituents of lavender essential oil, only monoterpene synthesis will be explained in detail here.

1.4.3. Monoterpene biosynthesis

Monoterpenes are derived from GPP by the activity of various monoterpene synthases (sometimes called cyclases). In the initial step GPP is ionized and isomerized to linalyl diphosphate (LPP) which is subsequently ionized and cyclized to yield the α -terpinyl cation (McGarvey and Croteau, 1995). The different monoterpene skeletons are derived from the latter highly reactive intermediate by further modification through specialized monoterpene synthases, cytochrome P450 hydroxylases, dehydroxygenases, reductases, glycosyl transferases or alkyl transferases.

Monoterpene synthases have been isolated and cloned from a number of plants including mint, lemon, snapdragon, sage, and *Arabidopsis* and even gymnosperms like grand fir (Alonso et al., 1992, Colby et al., 1993, Wise et al., 1998, Bohlmann et al., 1999, Bohlmann

et al., 2000, Luckner et al., 2002, Dudareva et al., 2003). An unusual feature shared by most monoterpene synthases is that these enzymes produce multiple products. For example, limonene synthase produces primarily limonene but also generates smaller amounts of myrcene and α - and β -pinene (McGarvey and Croteau, 1995). This product diversity indicates a common evolutionary origin for plant terpene synthases. In fact, all monoterpene synthases share similar properties, i.e. a native molecular mass ranging between 50-100kDa (either monomeric or dimeric), a requirement for a divalent metal ion as cofactor for catalysis (usually Mg^{2+} or Mn^{2+} for angiosperms, K^+ , Mn^{2+} , Fe^{2+} for gymnosperms), a pI near 5.0 and a pH optimum within a unit of neutrality (Bohlmann et al., 1998b). Monoterpene synthases are operationally soluble, but are found to be associated with plastids *in vivo* (Bohlmann et al., 1997). Sequence analysis of different terpene synthases from various plants revealed conserved sequence and structural characteristics, including amino acid sequence homology, conserved sequence motifs, equal intron number and position and similar exon size (Bohlmann et al., 1998b). A study by Bohlmann et al. (1998) on 33 selected monoterpene synthases from different plant species (angiosperms and gymnosperms) indicated four conserved amino acid motifs, i.e. the RRx8WD motif, LQLYEASFLL motif, DDxxD and (N,D)D(L,I,V)X(S,T)XXXE motifs. The RRx8WD motif is essential for the enzymatic activity of many monoterpene synthases (Williams et al., 1998) while the LQLYEASFLL motif is thought to be part of the active site (McGeady and Croteau, 1995, Wise et al., 1998). The DDxxD and (N,D)D(L,I,V)X(S,T)XXXE motifs are responsible for the enzymatic activity and coordination of divalent cations and thus responsible for substrate binding and ionization, respectively (Whittington et al., 2002, Christianson, 2006). Phylogenetic analysis discerned that terpenoid synthases (TPS) are categorized into six gene subfamilies (designated TPSa-TPSf). TPSa is constituted by sesquiterpene and diterpene synthases from angiosperms, TPSd is comprised of 11 gymnosperm monoterpene, sesqui- and diterpene synthases. TPSc, TPSe and TPSf are represented by single angiosperm terpene synthases, i.e. the diterpene synthases copalyl diphosphate synthase and kaurene synthase and the angiosperm linalool synthase, respectively. Many monoterpene synthases, including identified monoterpene synthases from *Lamiaceae* belong to the TPSb family (Bohlmann et al., 1998b, Trapp and Croteau, 2001).

1.5. Monoterpene storage and secretion

Where large amounts of hydrophobic terpenoids are produced and accumulated, specialized secretion structures are usually required. For instance, conifers have developed a species-specific system of cellular blisters and highly specialized ducts for the storage of resin, a mixture of volatile and non-volatile terpenes (McGarvey and Croteau, 1995). Terpenes are sometimes produced and stored in ‘traumatic resin ducts’ near the site of an injury or simply sequestered in proximity to the wound (McGarvey and Croteau, 1995). In angiosperms, monoterpene biosynthesis is often restricted to specific tissues at their site of utilization. Many flower fragrances, for instance, result from volatile terpenes, which occur in the form of minute droplets in the cytoplasm of the epidermal and neighbouring mesophyll cells of the sepals (Pichersky et al., 1994, McGarvey and Croteau, 1995).

In *Lamiaceae*, essential oil production and secretion are localized to specialized glandular trichomes (Figure 5) (Fahn, 1988, Lis-Balchin, 2002). Glandular trichomes are modified epidermal hairs that cover leaves, stems and parts of the flower. Two forms of glandular trichomes can be observed. The smaller, capitate glandular trichome consists of a basal cell, a short stalk and a one to two cell head (Fahn, 1988). Peltate glandular trichomes on the other hand are more complex. They are composed of eight secretory cells (disc cells), a stalk cell and a basal cell, anchoring the trichome in the epidermis (Figure 5) (Fahn, 1988). The outer surface of either gland is covered with a toughened cuticle usually completely covering the trichome. The essential oil accumulates in the subcuticular spaces that are formed by separation of the cuticle from the apical walls of the secretory cells. The exact secretion mechanism is still unclear, however, secretion of essential oil constituents is assumed to be achieved via diffusion of volatiles through the cuticle or by rupture of the cuticle (Fahn, 1988).

The quantity of monoterpenes produced is generally related to the age and size of the gland or by the density of glands per area of tissue. In peppermint, monoterpene synthesis and accumulation are controlled by the development of the oil glands during the growth season. Monoterpene abundance increases steadily as the glands increase in size, i.e. as glands pass through the one-, two-, four-, and eight-celled stages of their development (Turner et al., 2000a). Monoterpene abundance also increases during the season. Consistent with findings that the total number of peltate glands steadily increases during vegetative growth- the first leaves

produced in a season had 2,000 glands each; leaves of similar size, but 10 nodes younger (later in the season), had up to 17,000 glands per leaf (Colson et al., 1993)- essential oil abundance increased as the number of oil glands increased.

1.5.1. Compartmentalization within glandular trichomes

The ultrastructural features of mint trichomes strongly suggest that monoterpene synthesis is compartmentalized to specialized leucoplasts within glandular trichomes. Mature secretory cells of peltate glandular trichomes are characterized by enlarged leucoplasts surrounded by an extensive smooth endoplasmatic reticulum. These leucoplasts lack chloroplasts, grana and starch grains- a feature that renders these cells photosynthetically inactive (Turner et al., 2000a), but supports their function as the site of terpene synthesis. In contrast, glandular stalk cells develop distinctive plastids, numerous microbodies and abundant mitochondria and are likely to supply carbon substrate to the secretory cells (Turner et al., 2000a).

The compartmentalization of monoterpene biosynthesis to gland leucoplasts was further supported by the discovery of the plastidal DXP pathway and the localization of key terpene synthases to plastids. GPP synthase, an enzyme important in the early steps of monoterpene synthesis, is expressed as a preprotein bearing an N-terminal plastidal targeting sequence (Colby et al., 1993) and was directly localized to the leucoplasts of secretory gland cells by immunogold labelling (Turner et al., 1999). In addition, recent immunological studies demonstrated that four monoterpene synthases involved in menthol biosynthesis of peppermint are exclusively localized to secretory cells of glandular trichomes. However, enzymes involved in monoterpene biosynthesis are not exclusively found in leucoplasts. Localization of enzymes of the menthol biosynthetic pathway in mint showed that although synthases involved in early committed steps (GPP synthase) were localized to leucoplasts, enzymes catalyzing steps further downstream in the pathway were associated with the endoplasmic reticulum (ER) (limonene-6-hydroxylase), localized in the cytoplasm (pulegone reductase) or in mitochondria (isopiperitenol dehydrogenase) (Turner and Croteau, 2004). These findings were explained in a model proposed by Turner et al. (2004), in which primary monoterpenes, e.g. limonene, are synthesized in plastids, followed by their transport to the ER for further modification (Turner et al., 2000a, Turner and Croteau, 2004).

1.6. Regulation of monoterpene biosynthesis

1.6.1. Environmental regulation of monoterpene production

Secondary metabolites, like monoterpenes, hold a variety of ecological functions and are therefore regulated by their environment. Flowers produce the most diverse and the largest quantities of monoterpenoids just before the flower buds open, i.e. when the flower is ready for pollination. The released volatiles are suggested to serve or aid as pollinator attractants (Raguso and Pichersky, 1999). Terpenes also function as pathogen deterrents. For instance, fungal infection of castor bean seedlings resulted in increased levels of casbene, an antifungal and antibiotic diterpene of castor bean (Dudley et al., 1986). Indirect defence of plants against herbivores often involves the induced emission of volatile terpenoids possibly to attract natural enemies of the herbivores. Infestation of *Lotus japonicus* by two-spotted spider mites induced the emission of (E)- β -ocimene (Arimura et al., 2004a, Arimura et al., 2004b). Similarly, feeding forest tent caterpillars induced local and systemic diurnal emissions of (-)-germacrene D, along with (E)- β -ocimene, linalool, and (E,E)- α -farnesene from leaves of hybrid poplar (Arimura et al., 2004a).

Monoterpenes have also been shown to act as a protectant against high temperature stress (Velikova et al., 2006) and their emission is regulated by the level of irradiation in some plant species (Staudt and Seufert, 1995). In peppermint, a close relative of lavender, plant growth and oil yield were affected by photoperiod: short days resulted in decumbent plants, small leaves and many stolons, while long days, high photon flux density and high night temperatures favoured erect plants, large leaves and flowers, and highest essential oil yields (Clark and Menary, 1980b). Light intensity, day length and ambient temperature also influenced essential oil composition. Long days, high photon flux and cool nights favoured the accumulation of the more oxidized monoterpenes 1,8-cineol and menthone, while short days in combination with long warm nights led to an increase in the more reduced monoterpene menthofuran in peppermint (Clark and Menary, 1980a, Clark and Menary, 1980b).

1.6.2. Developmental regulation of monoterpene production

The monoterpene profile of a plant also changes during different stages of plant development. Dudareva et al. (2003) showed that the emission of β -ocimene and myrcene from snapdragon flowers was controlled by the developmental state: monoterpene emission was nearly undetectable in unopened and 1-day-old flowers, while emission increased strongly on the second day after anthesis and peaked 5-7 days after anthesis (Dudareva et al., 2003). In peppermint, monoterpene abundance is linked to the developmental stage of a leaf. Monoterpene abundance is comparably low in newly emerging leaves, but increases rapidly as the leaf expands. Monoterpene biosynthesis peaks between twelve and twenty days after leaf emergence, and then rapidly declines as full leaf expansion is reached (Turner et al., 2000b). Similarly, the total monoterpene content of peppermint leaves increases rapidly between days 12 and 20, levels off as full expansion is reached, and then remains stable for the remainder of the leaf life (Turner et al., 1999).

1.6.3. Regulation of monoterpene production through gene expression

To ensure that monoterpenes and their derivatives are available at all possible times, environmental conditions and developmental stages and thus can improve or grant the plant's survival, monoterpene biosynthesis and emission must be under strict regulation. Studies by Dudareva et al. (1996, 2003) showed that the abundance of mRNA, protein and enzymatic activity of β -ocimene synthase in snapdragon and S-linalool synthase expression in *Clarkia breweri* flowers were highly correlated with the emission levels of the corresponding monoterpene (Dudareva et al., 1996, Dudareva et al., 2003).

The close correlation of monoterpene synthase transcription, *de novo* monoterpene biosynthesis and monoterpene emission suggests that monoterpene production is regulated at the level of gene transcription (Dudareva et al., 1996, McConkey et al., 2000). In support of this hypothesis, Mahmoud and Croteau (2003) demonstrated that overexpression and cosuppression of the gene for menthofuran synthase, the enzyme catalyzing the oxidation of pulegone, resulted in the respective increase or decrease in the production of menthofuran in peppermint (Mahmoud and Croteau, 2001, Mahmoud and Croteau, 2003). On the other hand, overexpression of limonene synthase in peppermint leaves failed to increase the abundance of

the limonene synthase transcript and protein in oil glands. Accordingly, peppermint oil composition and yield were unaffected (Mahmoud et al., 2004). This lack of effect was reasoned to be due to the insufficient activity of the utilized promoter (CaMV 35S promoter) at the site of monoterpene synthesis (oil glands) and a potential lethal effect of strong ectopic expression of monoterpenes in leaf tissue (Mahmoud et al., 2004). Alternatively, these results may indicate that the production of some monoterpenes is controlled by other unknown mechanisms, or by the combination of transcriptional and other regulatory mechanisms.

1.7. Monoterpene abundance and regulation in lavender

The growing popularity of aromatherapy and alternative medicine has caused increasing interest and growing demand for lavender oil, triggering the development of new cultivation areas worldwide. The Okanagan valley (British Columbia) has become an important locale for small-scale, tourist-oriented lavender farming, which focuses on the marketing of ‘value-added oil’ for cosmetics, alternative medicine and aromatherapy.

Commercial distribution of lavender oil, especially for medicinal application, requires that oil quality adheres to highest standards, and regional variation in oil composition has to be kept to a minimum or at least has to be accounted for. Empirical data showed that monoterpene composition is highly variable among different lavender species as well as in different areas of cultivation (Lis-Balchin, 2002). To account for this variability, lavender oil from various species has been standardized by the International Organisation for Standardization (ISO) (Lis-Balchin, 2002). So far, little is known about the factors that cause variation in lavender essential oil or control the monoterpene profile and monoterpene abundance in lavender. Current knowledge stems mostly from empirical observations, gathered over decades of lavender farming, or is inferred from studies in related *Lamiaceae* species such as mint and sage. Lavender oil from France is considered superior to lavender oil cultivated elsewhere. (Lis-Balchin, 2002). At the species level, ‘English lavender’ (*Lavandula angustifolia*) is generally considered to produce the highest quality oil, characterized by negligible amounts of camphor and high levels of linalool but low overall oil yield. In comparison, *Lavandula x intermedia*, produces large amounts of low quality oil, marked by high camphor content (Lis-Balchin, 2002). However, which of these two factors- growth region or species- is the overriding factor that determines the monoterpene composition of lavender oil is unclear.

I hypothesized that the abundance of linalool and camphor is predetermined by the lavender species. To test this hypothesis, I determined the essential oil/monoterpene profile of several *L. angustifolia* and *L. x intermedia* cultivars grown in the Okanagan. The abundance of four major monoterpenes in various cultivars was compared between two lavender species cultivated under similar conditions as well as to internationally standardised lavender oil derived from these species.

Numerous studies have shown that monoterpene abundance and profile change throughout plant life. It was suggested that changes in monoterpene abundance are caused by the biological changes throughout plant development and/or due to differing climatic, edaphic or ecological conditions. **I hypothesized that monoterpene abundance in lavender is developmentally regulated.** To test this hypothesis, I measured monoterpene abundance throughout the development of vegetative and reproductive tissue and compared monoterpene accumulation (or loss) in two representative cultivars of *L. angustifolia* and *L. x intermedia*.

The mechanisms that regulate monoterpene abundance are largely unknown. In some cases a striking correlation between monoterpene content and monoterpene synthase expression has led to the idea that monoterpene abundance is controlled by monoterpene synthase expression. Therefore, **I hypothesised that monoterpene abundance is regulated at the level of gene transcription**, i.e. that monoterpene abundance correlates with the transcription of the respective monoterpene synthase. To test this hypothesis, I compared the transcription level of a previously reported linalool synthase from *L. angustifolia* as well as a putative linalool synthase from *L. x intermedia* to the abundance of linalool measured throughout the development of the respective lavender species.

Together, these results allowed the classification of Okanagan lavender oil and provided a basic understanding of monoterpene regulation in lavender. Increased knowledge of the distribution of monoterpenes between lavender species, tissues and during development will aid to limit essential oil variation and facilitate essential oil production by allowing growers to synchronize lavender harvest to the developmental state with the most favourable monoterpene profile or by regulating monoterpene synthase expression through genetic engineering.

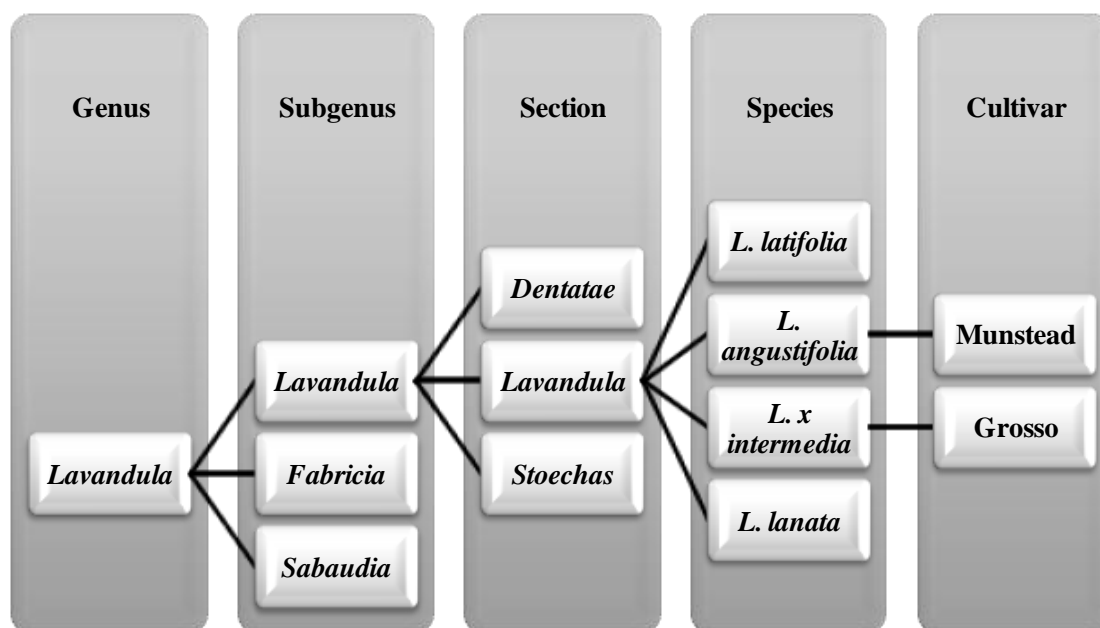
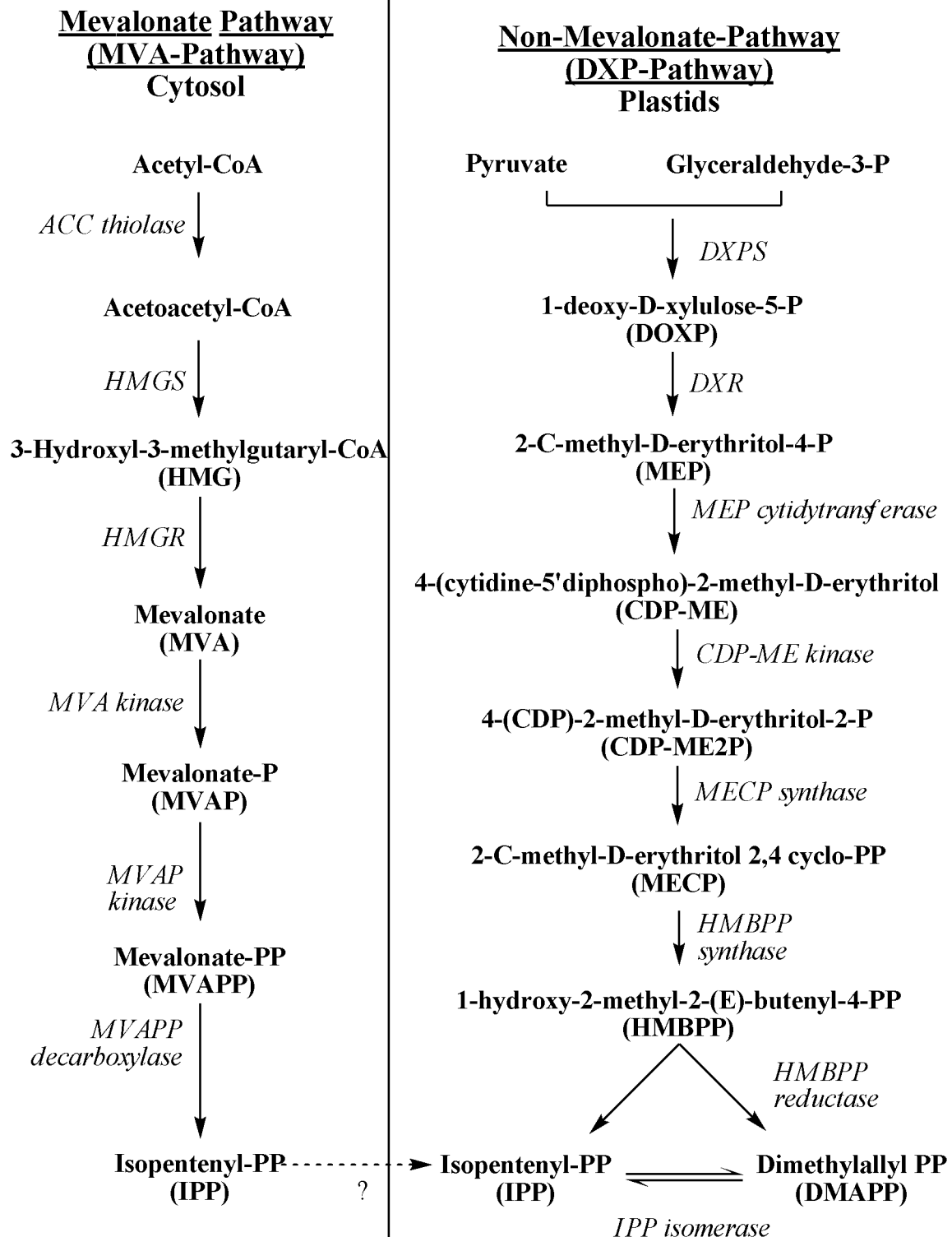


Figure 1: Subgenus, sections, species and cultivars within the genus *Lavandula*

Content (%) of major terpenes in lavender oil			
	English lavender	Lavandin	Spike lavender
Camphor	0.5-1%	4-11%	10-20%
Caryophyllene	3-12%	n.d.	n.d.
1,8-Cineole	1-2%	5-10%	20-30%
Linalool	30-49%	30-40%	40-50%
Linalool acetate	30-45%	20-30%	<1%
Ocimene	2.5-6%	n.d.	n.d.
Pinene (α and β)	n.d.	n.d.	1-3%

Table 1: Major mono- and sesquiterpenes in English lavender, Lavandin and Spike lavender (Lis-Balchin, 2002)

Figure 2: Biosynthesis of IPP and DMAPP via the mevalonate pathway (left) and the mevalonate-independent (DXP) pathway (right). The indicated enzymes are: AACT, acetyl-CoA/acetyl-CoA C-acetyl-thiolase; HMGS, 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; MVA kinase, mevalonate kinase; MVAP kinase, phosphomevalonate kinase; MVAPP decarboxylase, mevalonate-5-diphosphate decarboxylase; DXPS, 1-deoxyxylulose-5-phosphate synthase; DXR, 1-deoxyxylulose-5-phosphate reductoisomerase; MEP cytidyl transferase, 2-C-methylerythritol-4-phosphate cytidyltransferase; CDP-ME kinase, 4-(cytidine-5'-diphospho)-2-C-methylerythritol kinase; MECP synthase, 2-C-methylerythritol-2,4-cyclodiphosphate synthase; HMPPP synthase, 1-hydroxy-2-methyl-E-butenyl-4-diphosphate synthase; HMBPP reductase, 1-hydroxy-2-methyl-E-butenyl-4-diphosphate reductase and IPP isomerase (IPPI). The pathway may give rise to IPP and DMAPP independently of the interconversion catalyzed by IPPI. A transfer of IPP/DMAPP between cytosol and plastid is possible but, as of yet, unproven.



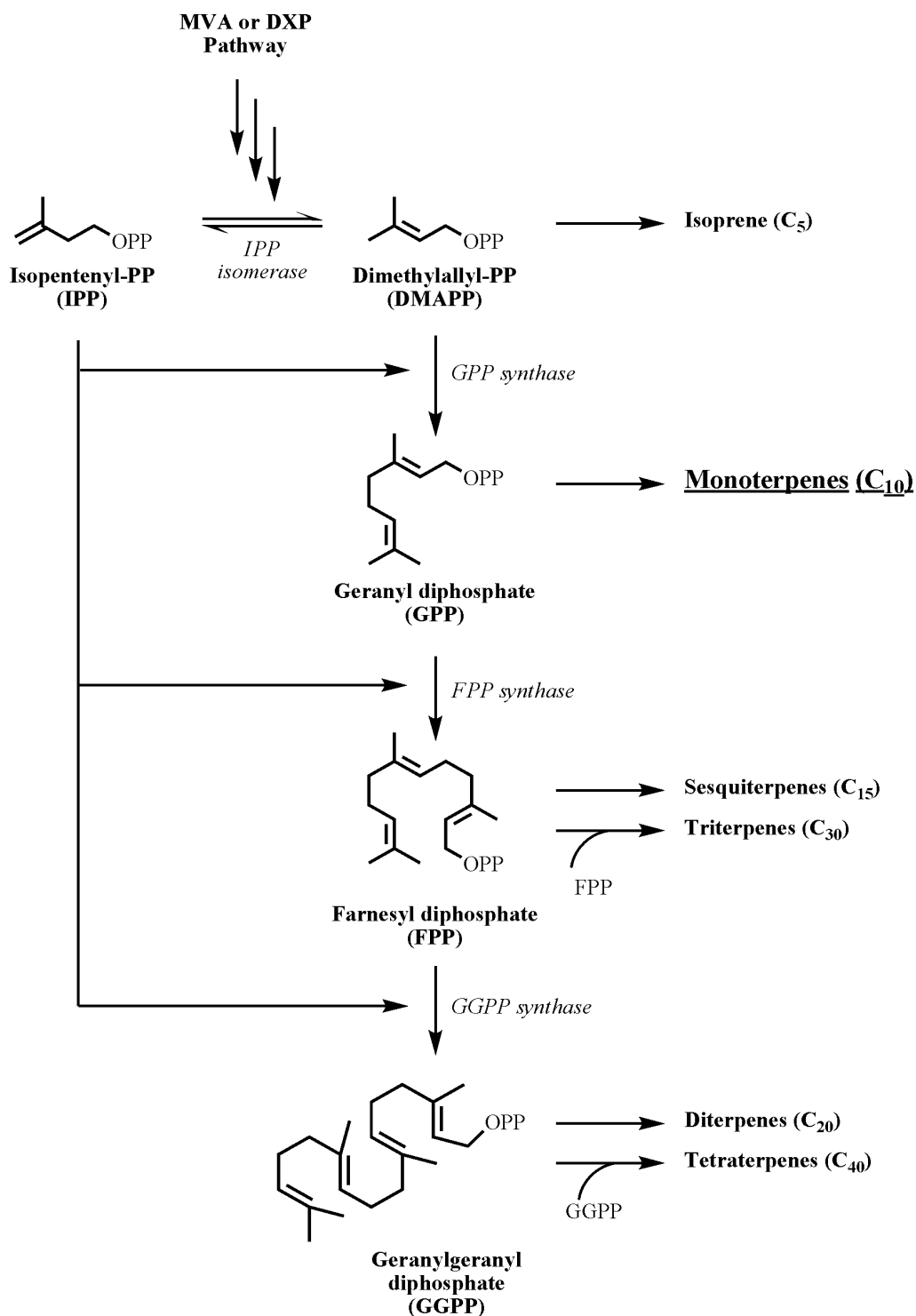


Figure 3: Terpene biosynthesis. GPP is synthesized by the condensation of one molecule of IPP and one molecule of DMAPP catalyzed by GPP synthase. FPP is the condensation product of GPP and one molecule of IPP, while GGPP is produced through the condensation of one molecule of GPP and two molecules of IPP. Monoterpenes are derived from the derivatization and rearrangement of GPP, while FPP and GGPP are the precursors to sesqui- and triterpenes and di- and tetraterpenes.

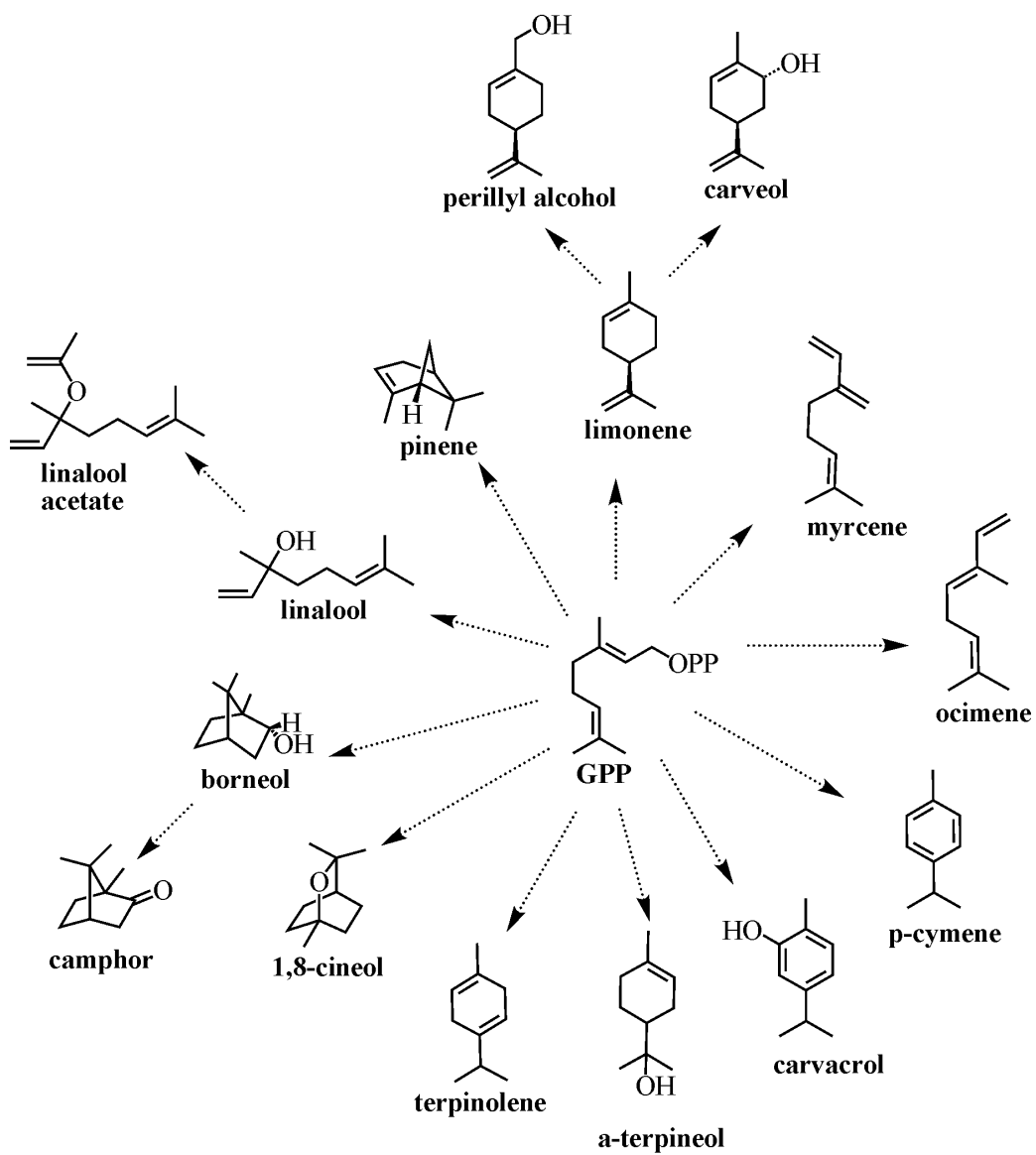
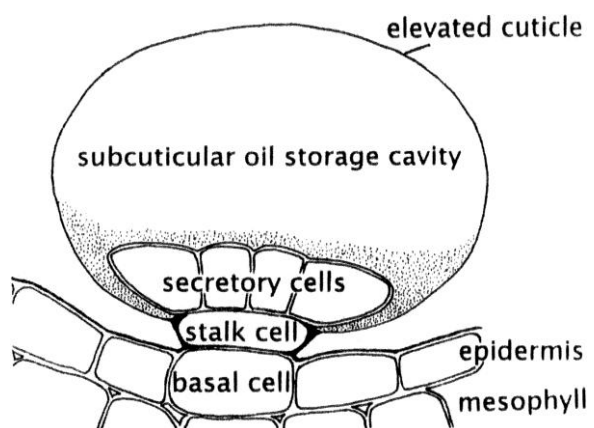
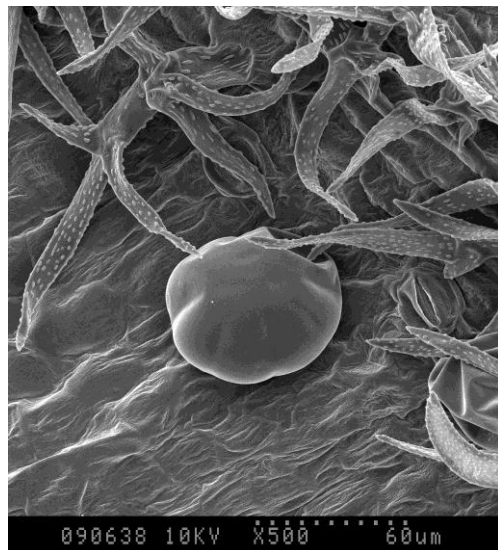
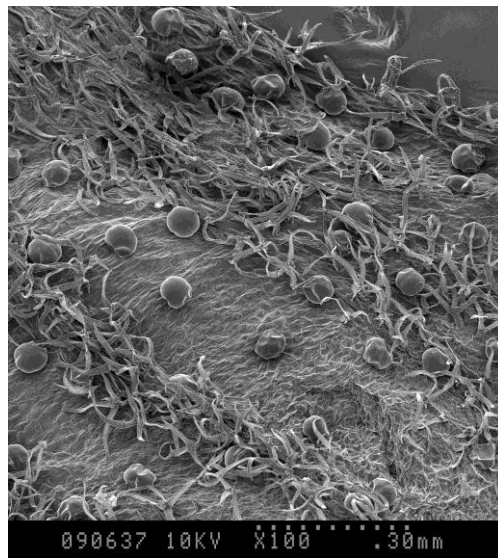


Figure 4: Selection of monoterpenes generated from geranyl diphosphate (GPP). Interrupted arrows indicate pathways with multiple possible intermediates.

Figure 5: Glandular trichomes. Upper panel: Scanning electron microscope (SEM) image of glandular trichomes on lavender leaves at 100x magnification. Middle panel: scanning electron microscope (SEM) image of a glandular trichome at 500x magnification. Lower panel: Schematic of a peltate glandular trichome from peppermint (Fahn, 1979) (Fahn, 1979, Turner and Croteau, 2004). SEM images courtesy of Michael Weis, Electron Microscopy & Digital Imaging, Pacific Agri-Food Research Centre, Agriculture and Agri-Food Canada, Summerland, BC



2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All chemical and reagents used were of analytical purity grade. Unless otherwise stated, biochemical reagents and organic solvents were obtained from Sigma-Aldrich (Canada) and Fisher Scientific (Canada). Restriction enzymes, T4-DNA ligase, DNA-polymerases, desoxynucleotide-tri-phosphates (dNTPs) and corresponding buffer solutions were purchased from Invitrogen (Canada), Fermentas Life Sciences (Canada), Novagene (Canada), Qiagen (Mississauga, Canada) or New England Biolabs (Ipswich, USA).

2.2. Bacteria and plasmids

The suppliers and genotypes of bacterial strains used in this study are listed in Table 2. Plasmids used were pCR®8/GW/TOPO (Invitrogen, Canada) for cloning of PCR fragments and pET32a (Novagen, Canada) for heterologous protein expression.

2.3. Plant material

L. angustifolia and *L. x intermedia* plants (*L. angustifolia* cv. Bowles, Hidcote, Lavender Lady, Mailette, Munstead, Premier, Royal Velvet, Royal Purple; *L. x intermedia*, cv. Grosso, Hidcote Giant, Super) were grown under natural conditions at a field site at the University of British Columbia Campus (Kelowna, BC, Canada). The site was located at approximately 49° 57' N latitude, 119° 24' W longitude and an elevation of approximately 450 m. According to recommendations by local growers, the site was prepared with top soil mixed with 10% steer manure, and approximately 5g of bone meal per plant as initial fertilization. The site was overlaid with heavy duty, black landscape fabric before lavenders were planted. Individual plants were spaced 1 m apart, watered every two days for 10 min and fertilised biannually (March, October) with Miracle-Gro all purpose fertilizer (15:30:15 N:P:K ratios, The Scotts Company, Marysville, OH, USA).

2.4. Plant harvest

At the time of harvest, plants were 2 years of age. Flowers and leaves were sampled from mid June to mid September during the flowering season. For comparative essential oil analysis, entire flower spikes of *L. angustifolia* and *L. x intermedia* were harvested when approximately 30% of the individual flowers per spike were in bloom. Tissue samples from 3 individual plants were combined and frozen at -80°C immediately after harvest.

Grosso and Munstead flowers at different stages of development were selected according to spike size (length of the spike in cm), colour (green or violet) and number of flowers post anthesis per flower spike. Seven different stages were distinguished as described in Table 3 and 4. Leaves of three developmental stages were distinguished according to size of the leaf blade and position of the leaf. The youngest leaves were collected from the first node below the flower spike; leaf blades were no larger than 2 cm. Intermediate aged leaves included fully expanded leaves (4-5 cm) collected from the 6th-8th node of the flower stalk (peduncle), while fully expanded leaves, sampled from the body of the lavender (from woody plant parts) and a clearly distinguishable darker colour, represented developmentally oldest leaves. For essential oil extraction, tissues from three individual plants were combined and frozen at -80°C immediately after harvest. For RNA extraction, tissues from three individual plants were combined and shock frozen in liquid nitrogen immediately after sampling to prevent RNA degradation, and stored at -80°C.

2.5. Essential oil analysis

This study was initially designed to determine which cultivars had high camphor content, and with the intent to further analyse camphor biosynthesis in high camphor expressing varieties at the molecular level. For this purpose, essential oil was extracted from twelve lavender cultivars, and the method was adjusted to measure the relative camphor content (%) in these oils. However, during the course of the project the objectives were expanded to determine also the absolute amount of camphor as well as relative and absolute abundances of limonene, linalool, linalool acetate and borneol, in order to allow inferences about the biosynthesis of these compounds, their relationship and their correlation to the expression of isolated

monoterpene synthases. The method for essential oil analysis was tested for the accuracy in measuring these additional compounds; however, due to time constraints, the method was not further optimized for the quantification of compounds for which the method was suboptimal.

2.5.1. Essential oil extraction

Essential oil was extracted from frozen lavender leaves and flowers by simultaneous steam distillation solvent extraction (SDSE) using a Likens-Nickerson type apparatus equipped with a standard condenser (Figure 6) as described previously (Mahmoud and Croteau, 2001, Mahmoud and Croteau, 2002). The procedure was optimized for the extraction of monoterpenes from lavender tissue, i.e. the optimal solvent and solvent volume were determined prior to sample extraction. For optimal extraction, frozen tissue of 3-4 individual plants was pooled and crushed manually to ensure sample homogeneity. Five to ten grams of the pooled sample were heated to reflux in flask A (Figure 6) with 90 ml of distilled water and 1 mg of menthol as internal standard. The organic phase, consisting of 15 ml of pentane was heated in flask B (Figure 6). The volatile monoterpenes which were released from the boiling sample, mixed and solubilized in the pentane vapor. The vapor was cooled and condensed in a condenser which was kept at zero degrees by ethylene glycol. Both water and pentane were heated for 45 min after reflux had begun. The pentane layer was collected and stored at -20°C until Gas Chromatography/Mass Spectrometry (GC/MS) analysis. The essential oil extraction was repeated twice for each pooled sample.

2.5.2. Gas Chromatography / Mass Spectrometry (GC/MS) analysis of essential oil

Distillation extracts were diluted 1:100 with pentane for flower samples or used undiluted for less concentrated leaf extracts and analyzed by GC/MS. GC analysis was performed using a Varian GC 3800 gas chromatograph equipped with polyethyleneglycol ester capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness, ECTM 1000, Alltech, Deerfield, IL). One µl of each sample was loaded onto the column in split mode (20:1). The temperature programming was initiated at 40°C for 3 min, increased to 170°C at 7°C/min, and to 230°C at 30°/min. The injector temperature was held at 250°C; helium was used as a carrier gas at a flow rate of 1 ml/min. Response peaks above a cut off minimal response of 500 counts were recorded and the peak area was integrated. GC eluates were analyzed in a Saturn 2200 Ion Trap

mass detector operating in EI mode at 70 eV. Mass spectra were recorded within 40-650 (m/z) full scan mode. Each sample was analyzed twice. Monoterpene content was reported as the average of two extractions multiplied by two analytical replicas of each pooled tissue sample.

2.5.3. Monoterpene identification

Individual monoterpenes were identified by comparing their retention times to the retention time of pure authentic standard whenever possible. In addition, each compound was analyzed in an ion trap mass spectrometer. The sample mass spectrum was compared to standards cataloged by the National Institute of Standards and Technology (NIST) and a specialized essential oil library (library for the identification of essential oil components by GC-‘quadrupole’-MS, R.P. Adams). Mass spectrum comparison served as a secondary confirmation for compounds with known retention times or as a primary identifier for compounds for which authentic standards could not be obtained and thus retention times were missing.

2.5.4. Monoterpene quantitation

2.5.4.1. Relative monoterpene abundance

The relative amount (percentage) of each monoterpene was estimated by dividing the peak area of the compound of interest by the sum of all peaks detected (Peak area compound of interest/Sum of all peak areas x 100 = % compound of interest in sample). Relative monoterpene amounts were averaged for two extraction- and three instrumental replicas.

2.5.4.2. Absolute monoterpene abundance

The absolute abundances (as ng/μl) were calculated based on a four point calibration curve, using a mixture of authentic standards diluted to 0.5 ng/μl, 5 ng/μl, 50 ng/μl, and 500 ng/μl each. A simple linear regression by the least squares showed that the compound peak response was linear over the selected concentration range, with correlation coefficients of $r^2=0.99$ (limonene), $r^2=0.99$ (camphor), $r^2=0.98$ (linalool), $r^2=0.98$ (linalool acetate) and $r^2=0.99$ (borneol). Measured abundances in each extract were normalized to the amount of tissue initially extracted (monoterpene/gfWT). Absolute monoterpene amounts were averaged for two extraction- and three instrumental replicas.

2.5.4.3. Total oil abundance

Total essential oil concentration (as mg/gfWT) was calculated by integrating the area of all compound peaks in the sample and correlating the sum of peak areas to the peak area of the internal standard (menthol at 1 mg), assuming a unit response for all compounds. The total essential oil concentration was normalized to the amount of tissue in the extraction ($\text{Sum of peak area} \times 1 \text{ mg/area of menthol peak} / \text{gfWT} = \text{mg of essential oil per gfWT}$).

For the sake of practicality, calculation of total oil abundance disregarded any loss of menthol standard during the extraction process and assumed equal peak responses among all compounds, i.e. assumed that all compounds produced equal peaks at equal concentrations. However, in practice, the peak response differed among compounds. Therefore, total essential oil amounts represent merely a relative estimate of the actual essential oil content.

2.5.5. Accuracy of monoterpene quantitation

The accuracy of monoterpene quantitation was tested based on four parameters, i.e. limit of detection, limit of quantitation, detection efficiency of monoterpenes across the working concentration range, and monoterpene stability.

2.5.5.1. Limit of detection and quantitation

The limit of detection (LOD) - the lowest concentration level that can be determined to be statistically different from a blank - was established for borneol, camphor, limonene, linalool and linalool acetate using serial dilutions of authentic standards (0.01 ng/μl to 1000 ng/μl in increments of one order of magnitude). The lower limit of detection for borneol, camphor, linalool and limonene was 0.01 ng/μl, with a signal to noise ratio of ~4:1. The lower limit of quantitation (LOQ)- the level at which the signal to noise ratio was ~10:1 and quantitative results could be obtained- was 1 ng/μl for these four compounds. For unknown reasons, the limit of detection for linalool acetate was substantially higher (0.5 ng/μl), as was the limit of quantitation (5 ng/μl) for this compound.

2.5.5.2. Detection efficiency

Detection efficiency was based on the monoterpene recovery, i.e. the proportion of monoterpene that could be detected in a sample of known concentration. Monoterpene

recovery was measured for a mixture of borneol, camphor, limonene, linalool and linalool acetate at five different working concentrations (0.5, 5, 50, 500 and 5000 ng/μl). Recovery of borneol, camphor, limonene and linalool was most accurate at initial concentrations between 0.5-500 ng/μl (recovery of 77%-108%). Highest recoveries (98%-108%) were obtained at an initial concentration of 50 ng/μl, while monoterpene recovery for concentrations higher than 500 ng/μl was lowest ($\leq 50\%$), a fact that can be explained by poor peak resolution and peak trailing at high concentrations. Linalool acetate recovery was considerably lower, especially at lower concentrations (58%-60% at an initial concentration of 0.5-50 ng/μl). Accurate quantitation of this compound could only be achieved at 500 ng/μl (96% recovery).

2.5.5.3. Monoterpene stability

The monoterpene stability, i.e. the duration over which the monoterpene abundance in a sample extract was constant, was measured for (standard) mixtures of borneol, camphor, limonene, linalool and linalool acetate at five different working concentrations (0.5, 5, 50, 500 ng/μl), spanning the concentration range for which accurate quantitation of these compounds was obtained. The mixture was stored at -20°C, and monoterpene abundance was measured at six different times within a 24 day period. Measurements were normalized to the amount of monoterpene detected in the non-stored mixtures to account for changes in monoterpene recovery due to concentration dependent detection efficiency (as demonstrated above). Within 24 days, borneol, camphor, limonene and linalool decreased by up to 34% depending on the initial concentration, while linalool acetate decreased by an average of 51%. Highest monoterpene recovery and least loss (0%-12%) were obtained at an initial concentration of 5 ng/μl, except for linalool acetate which could not be detected at this concentration.

2.5.6. Limitations of monoterpene quantitation

Based on the detection efficiency and recovery during storage, accurate quantitation was achieved at a sample concentration between 5 ng/μl-500 ng/μl for borneol, camphor, limonene and linalool, and 500 ng/μl for linalool acetate. A 100-fold dilution of the extraction sample moved the in-sample concentration of most of the observed monoterpenes into the anticipated concentration range. However, the concentration of monoterpenes in lavender extracts ranged from 0.5-1500 ng/μl (and thus exceeded the target range); thus, optimal

quantitation at this dilution could only be maintained for borneol, camphor, limonene and linalool, while quantitation of linalool acetate was unreliable. Accordingly, linalool acetate was omitted from further analysis.

2.6. Statistical analysis

Statistical analysis was done using the SPSS 7.0 software package at an $\alpha=0.05$ level. The average percentages (averages of two extractions times two analytical replicas of each pooled tissue samples) of borneol, camphor, linalool and limonene in eight *L. angustifolia* cultivars (n=8) were compared to the average abundances of these monoterpenes in three *L. x intermedia* cultivars (n=3). Monoterpene percentage measured in the 11 different cultivars was the dependent variable; lavender species was determined as the independent variable (*L. angustifolia* or *L. x intermedia*). The data set was tested for normality using the Shapiro-Wilk Goodness of Fit test. All monoterpene abundances were non-normally distributed. The measured monoterpene percentages were therefore converted to a ranked dataset and analyzed using the non-parametric equivalent to ANOVA, i.e. the Kruskal-Wallis test. The test tested the null hypothesis that mean ranks of borneol, camphor, linalool and limonene percentages did not substantially differ between *L. angustifolia* and *L. x intermedia* cultivars.

The Spearman Rho correlation coefficient was determined to test the relationship between transcript- and monoterpene abundance. Therefore, average transcript abundance was compared to average linalool abundance (ng/gfWT).

2.7. Isolation and reverse transcription of messenger RNA

Total RNA was isolated from *L. x intermedia* cv. Grosso or *L. angustifolia* cv. Munstead tissue (leaves, flowers of different developmental stage) using the RNeasy Plant Mini Kit (Qiagen, Mississauga, Canada) according to the manufacturer's instructions. Messenger RNA was reverse transcribed into first strand cDNA with Superscript II reverse transcriptase and a commercial oligo-dT primer.

2.8. Polymerase Chain Reaction (PCR)

A basic PCR reaction generally contained 0.2 mM of each adenosine-, thymidine-, guanine- and cytosine desoxytriphosphate (dNTP), 1.5 mM MgCl₂, 1 µM forward primer, 1 µM reverse primer, 1-20 µl (approximately 0.5ng-1µg) of DNA template (a single colony for colony-PCR), 1-2.5 U *Taq* DNA polymerase, buffered with 20 mM Tris-HCL, 50 mM KCl (1 x *Taq* buffer) in a 50 µl aqueous solution. The PCR cycle was initiated at 95°C for 3 min to completely denature the template, followed by denaturation at 94°C for 30 sec, annealing at 58°C for 1 min and extension at 72°C for 1 min for every 1000 base pairs. The denaturation-, annealing-, extension cycle was repeated 30 times, and finalized with a final extension at 72°C for 7 min. Samples were stored at 4°C until further use.

2.8.1. Gradient PCR

To determine the optimal annealing temperature for the amplification of selected monoterpene synthase cDNAs identical reaction samples (composed as described for a general PCR reaction) were amplified using different annealing temperatures as determined by the gradient setting of the thermocycler. The annealing temperature at which the highest abundance of single product was obtained was chosen as the optimal annealing temperature.

2.8.2. Reverse Transcriptase PCR (RT-PCR)

Reverse transcriptase PCR is a procedure by which messenger RNA is reverse transcribed into a stable complementary DNA (cDNA). The procedure consists of two consecutive steps: i) isolation of total RNA from tissue ii) reverse transcription of the mRNA into single strand copy cDNA, using Oligo(dT) primers that are targeted against the mRNA-specific poly-A-tail. In subsequent PCR, a gene of interest is amplified from this pool of single stranded copies of the transcript using gene specific primers.

Total RNA was isolated as described above. mRNA was reverse transcribed using SuperScriptTM II RT reverse transcriptase (Invitrogen) according to the supplier's recommendations. In short, 1 µl of 100 µM Oligo(dT)12-18, 1 µg of total RNA and 1µl of dNTP Mix (10 mM each) were mixed with sterile, distilled water to a final volume of 12 µl.

The mixture was heated to 65°C for 5 min and quickly chilled on ice before adding 5 x First-Strand Buffer and 4 µl of 0.1M DTT. The tube was incubated for 2 min at 42°C. One microlitre (200 units) of SuperScript™ II RT and distilled water (to a 20 µl final volume) were added and mixed by pipetting. The reaction mixture was incubated at 42°C for 50 min and the reaction was finally inactivated by heating at 70°C for 15 min. The cDNA product was directly used as template for amplification in PCR, assuming equal amounts of initial template and final product (assuming that 1 µg of total RNA will produce 1 µg of cDNA).

2.8.3. Semi-quantitative PCR

Quantities of *L. angustifolia* linalool synthase (*LIS*) and *L. x intermedia* (*Lxi-LIS*) mRNA were estimated by semi quantitative reverse transcriptase PCR. Therefore, 1 µg of total RNA was reverse transcribed into cDNA as described above. Five microlitres (5µg) of the first strand cDNA were used as template in a subsequent PCR reaction. *LIS* and *Lxi-LIS* cDNAs were amplified using gene specific primers: 5'- ATGTCGATCAATATCAACATGCC -3' (*LIS* forward), 5'- TCATGCGTACGGCTCGAACAGC -3' (*LIS* reverse). To ensure that equal amounts of RNA were used throughout samples and that RT reactions were equally effective, a control PCR was simultaneously performed with primers against *L. angustifolia* β-actin (5'- AGGCCAATCGTGAGAAGATG-3' (actin forward) and 5'- AAGGATTGCATGAGGGAGTG-3' (actin reverse)). The temperature programming was set to 2 min at 94°C, followed by 35 cycles beginning at 94°C for 30 sec, annealing at 64°C (*LIS* and actin primers) for 45 sec and elongation at 72°C for 2 min. The abundance of PCR products was compared visually after agarose-gel-electrophoresis.

2.8.4. Real-Time PCR

Transcript abundance was estimated by Real-Time PCR using the relative quantification system for Applied Biosystems 7300/7500 Real-Time PCR system and Qiagen QuantiTect SYBR Green PCR kit (Qiagen, Mississauga, Canada). First strand cDNA (250ng) was mixed with 2 x SYBR green master mix and 0.3 µM forward and reverse primer as recommended by the supplier (Qiagen, Mississauga, Canada)), in a total volume of 25 µl. *LIS* and *Lxi-LIS* transcription was measured using primers 5'- ATGTCGATCAATATCAACATGCC -3' (*LIS* forward), 5'-

TCATGCGTACGGCTCGAACAGC -3' (LIS reverse). The PCR reaction was activated at 95°C for 15 min, followed by forty cycles of denaturation at 94°C for 15 sec, annealing at 64°C for 30 sec and extension at 72°C for 2 min. The reaction was terminated by a final extension for 5 min at 72°C. A melting curve analysis (5 min at 58°C) of the PCR product(s) was performed subsequently to verify product specificity and identity.

LIS and actin transcript abundances were measured in three replicate samples for each tissue. *LIS* transcript numbers were calculated based on a five point calibration curve generated for a 191 bp portion of *L. angustifolia* β -actin (primers as above). *LIS/Lxi-LIS* transcription was expressed as a percentage of the abundance of actin transcript in each tissue.

2.9. Isolation and amplification of monoterpene synthase genes

A linalool synthase gene, endogenous to *L. x intermedia*, were identified and isolated using 'homologous' PCR (hPCR). Therefore, primers were designed homologous against the full length mRNA sequences of two previously identified linalool synthases (*LIS*) derived from either *L. angustifolia* (accession ABB73045, 5'- ATGTCGATCAATATCAACATGCC -3' (LIS forward), 5'- TCATGCGTACGGCTCGAACAGC -3' (LIS reverse)) or *L. latifolia* (accession ABD77417, 5'-ATGTCTATCATTAGCATGCATGTGG-3' (LIS forward), 5'-TTAGGGATATGGCTCGAACATCAGG-3' (LIS reverse)). The hPCR reaction contained 5 μ l of 10 x High Fidelity PCR buffer, 0.2 mM of each dNTP, 2 mM MgSO₄, 0.2 μ M of forward and reverse primer, 2 μ l of first strand cDNA (50ng), 1U Platinum® *Taq* High Fidelity and autoclaved, distilled water to 50 μ l. The PCR temperature programming was initiated with 2 min at 94°C, followed by 30 cycles of 94°C for 30 sec, annealing at 64°C and elongation at 72°C for 1 min per 1000 base pairs.

2.10. Agarose gel electrophoresis

Solutions containing DNA fragments (PCR reaction, DNA digest reaction, see 2.18) were applied to a 1.0% agarose gel containing 10 μ l of SYBR® Safe per 100 ml of 1% agarose and electrophoresed at 100V for approximately 1 hour. DNA fragments were visualized by UV illumination (365 nm). The size of each DNA band was estimated by comparison with a standard 1 Kb DNA ladder (Invitrogen).

2.11. Purification of DNA fragments

2.11.1. Column purification

To remove enzymes, primers, nucleotides and other impurities which might interfere with subsequent cloning steps, PCR samples were purified using the QIAquick PCR Purification Kit. Purified PCR fragments were eluted from the column with 50 µl of deionised water; final yield of the purified PCR fragments was estimated by agarose gel electrophoresis.

2.11.2. Purification of DNA by agarose gel electrophoresis

DNA fragments were purified from the agarose using the QIAquick gel extraction kit (Qiagen) according to the supplier's instructions. In principle, the DNA band of interest was excised from the agarose gel, dissolved in 3 volumes (w/v) of buffer QG at 50°C. This solution was mixed with 1 volume (w/v) of isopropanol and applied to the QIAquick column. After binding of the DNA to the silica matrix, traces of agarose were removed with 0.5 ml of buffer QG and washing with 0.75 ml of buffer PE. The DNA was eluted from the column with 50 µl of dH₂O and stored at -20°C until used further.

2.12. Ligation

Vector and insert DNA (PCR fragment or restriction fragment) were mixed in a ratio of 1:5 (vector: insert) and incubated with 10% (v/v) of 10 x T4 ligase buffer and 5% (v/v) T4 ligase in an aqueous solution at 4°C overnight. This ligation mixture was incubated for 5 min at room temperature (pCR®8/GW/TOPO vector backbone) or 30 min to over night at 16°C (pET32a vector backbone). After incubation, ligation samples were used directly in subsequent transformation reactions.

2.13. Transformation of *E. coli* DH5 α /DH10B

2.13.1. Transformation by heatshock

Twenty microliters of either *E. coli* Top10, BL21 (DE3) or Origami (DE3) chemically competent cells (Invitrogen, Novagen) were mixed with an aliquot of above ligation reaction or purified plasmid DNA and incubated on ice for 5 min. Cells were heatshocked for 45 sec at 42°C, iced for an additional 2 min and subcultured in 80-250 μ l of SOC medium (2% (w/v) Bacto-trypton, 0.5% (w/v) Bacto-yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM glucose, pH 7) at 37°C for 1 hour. Ten to fifty microliters of the bacterial culture were spread on LB-agar (Luria Bertani-broth (5% (w/v) NaCl, 5% (w/v) yeast extract, 10% (w/v) Bacto tryptone, 1.2% (w/v) agar-agar) plates containing the appropriate selective antibiotic (100 mg/l of ampicillin or 25 mg/ml of spectinomycin). Plates were incubated overnight at 37°C, and single colonies were selected for colony analysis.

2.13.2. Transformation by electroporation

One hundred microliters of electro-competent DH10B *E. coli* cells were mixed with 5-10 μ l of the ligation reaction solution (or isolated plasmid) and transferred to a chilled electroporation cuvette. The mixture was subjected to an electric pulse of 2.5 kV (capacitance at 25 μ F and 200 Ohm), and immediately mixed with 80-250 μ l of SOC medium. The transformation reaction was incubated for 1 h at 37°C and 5-10 μ l were plated on LB plates containing the appropriate antibiotic (100 mg/l of ampicillin or 25 mg/ml of spectinomycin) for selection. Plates were incubated over night at 37°C, and single colonies were selected for colony analysis.

2.14. Preparation of electro-competent DH10B

DH10B cells were grown in 100 ml of liquid LB medium until OD₆₀₀= 0.4-0.6. Cells were harvested by centrifugation at 4°C and resuspended in 50 ml of cold water. Cells were washed once in 50 ml of cold water, harvested and resuspended in cold, 10% glycerol. Cells

were harvested one last time, resuspended in 3 ml of cold 10% glycerol and stored as 80 µl aliquots at -80°C.

2.15. Transformation analysis

2.15.1. Colony PCR

Positive transformation of *E. coli* cells was determined by hotstart/colony PCR as described by Sambrook and Russell (2001). Therefore, a number of colonies grown on the LB plates were picked with a sterile tip and directly dipped into a solution containing 2.5 µl of 10x Taq-buffer, 1 µl of 10 mM dNTPs, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of Taq polymerase (Promega) and 18.5 µl of sterile water. Cell lysis was efficiently accomplished by the initial step of the PCR (5 min at 95°C). PCR was run as outlined for a general PCR reaction. Additionally, picked colonies were also dipped into culture medium (5 ml of LB, 100 mg/l of carbenicillin) and incubated at 37°C overnight for amplification of transformants. Positive transformants were identified by analysing the PCR samples by agarose gel electrophoresis. Those transformants with a positive insertion (DNA band with desired size detected on agarose gel) were selected to be sequenced.

2.15.2. Restriction analysis

Positive transformation was confirmed after isolating the plasmid and digesting it with a set of restriction enzymes (as described below). Restriction fragments were separated by agarose gel electrophoresis and the restriction fragment pattern was compared to the expected pattern.

2.16. Plasmid purification

2.16.1. Spin column purification

Plasmid DNA was purified from overnight cultures using the QIAprep spin miniprep kit (Qiagen) as described by the manufacturer or by alkaline lysis. Plasmids purified by this procedure are generally free of interfering salts and impurities and were thus used for subsequent sequencing reactions.

2.16.2. Purification by alkaline lysis

For purification by alkaline lysis, bacterial cells were collected from 1.5 ml of overnight culture by centrifugation. Cells were resuspended in 300 µl of TENS (0.1 N NaOH, 0.2% SDS, 10 mM Tris pH 7.5, 1 mM EDTA, 0.25 µg/µl RNase), which caused cell lysis. Cell debris and chromosomal DNA were precipitated by addition of 150 µl of 3 M sodium acetate, pH 5.2, and removed from the solution by centrifugation. Ninety-five percent ethanol was then used to precipitate the plasmid DNA from the supernatant, the supernatant was removed, and the DNA was resuspended in water. Since alkaline lysis may introduce nicks in the plasmid DNA, these preparations were only used for cloning or restriction analysis.

2.17. General restriction digest

Three to five micrograms of purified plasmid DNA were digested in a reaction containing the appropriate concentration of restriction enzyme buffer (as recommended by the supplier), plus 20-60 U of restriction enzyme in a 20 µl aqueous reaction.

2.18. Restriction digest of monoterpene-synthase vector plasmids

A general restriction digest contained 1-5 µg of the plasmid (pAB003, pAB007, pET32a or corresponding progeny plasmids), 20-60 U of EcoRI and 10% (v/v) of 10x EcoRI buffer (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 0.025% Triton X-100, pH 7.5 @ 25°C) in an aqueous solution. Digests were carried out overnight at 37°C; the digested plasmid was purified by agarose gel electrophoresis and stored at -20°C until further use.

2.19. Sequence analysis

Purified plasmid DNA or PCR fragments were sequenced by a commercial sequencing service (Plant Biotech Institute, Saskatoon or Fragment Analysis and DNA Sequencing Services (FADSS) at UBC Okanagan, Kelowna). The sequences of putative *LIS* gene and translation thereof were compared to the *L. angustifolia* and *L. latifolia* *LIS* gene/protein using the vector NTI sequencing package (Invitrogen).

2.20. SDS-Polyacrylamide-gel-electrophoresis (SDS-PAGE)

2.20.1. Preparation of SDS-polyacrylamide gels

SDS-polyacrylamide gels (SDS gels) were prepared according to Laemmli (Laemmli, 1970). For 12% SDS gels, the separating gel contained 0.375 M Tris/HCl pH 8.8, 12% (v/v) acrylamide/bisacrylamide and 0.1% (v/v) SDS in an aqueous solution, as well as 0.05% (w/v) ammonium persulphate and 0.1% (v/v) tetramethylethylenediamine (TEMED) as cross linking agents. The separation gel was overlaid with the stacking gel consisting of 0.125 M Tris/HCl pH 6.8, 3.9% (v/v) acrylamide/bisacrylamide, 0.1% (v/v) SDS, 0.05% (w/v) ammonium persulphate, 0.1% (v/v) TEMED and prepared in a procedure analogous to the stacking gel.

2.20.2. Sample preparation for SDS-gel electrophoresis

Protein solutions were diluted with equal volumes of 4 x SDS sample buffer (200 mM Tris pH 6.8, 40% (v/v) glycerol, 8% (v/v) SDS, 10% (v/v) dithiotreitol, 0.5% (w/v) bromophenolblue), denatured at 96°C for 5-10 min and separated by SDS-gel electrophoresis.

2.20.3. SDS-Gel-electrophoresis

Twenty to 30 µl of protein sample were loaded onto 12% SDS-gels, buffered with 1.5% Tris, 0.3% glycine, 1% SDS and run at a constant current of 25 mA for approximately 1.5 h.

2.20.4. Coomassie™ staining

After SDS-gel-electrophoresis, proteins were simultaneously fixed within the gel and stained in a solution containing 45% methanol, 0.45% Coomassie™ Brilliant Blue R-250 (CBB) and 10% glacial acetic acid for a minimum of 3 h. The gels were destained for 1-3 h with a solution of 30% methanol, 10% glacial acetic acid, which removed the CBB stain from the polyacrylamide background and left only protein bands stained.

2.21. Cloning and expression of linalool synthase

A putative *Lxi-LIS* cDNA was amplified by RT-PCR from *L. x intermedia* tissue as described above. The success of the PCR reaction was tested by agarose-gel-electrophoresis. Therefore, an aliquot of the PCR reaction sample was run on a 1% agarose gel. PCR reactions generally generated a single product of the expected size (1700bp for LIS), which was purified using Qiagen PCR purification columns as described by the supplier. The purified single PCR fragment of *Lxi-LIS* was subcloned into pCR®8/GW/TOPO vector (Invitrogen) (a vector for the amplification of the introduced gene, necessary for subsequent gene sequencing), utilizing the Taq-generated TA overhangs for facilitated cloning (Figure 7). Vector constructs were transferred into *E. coli* DH5α cells for amplification. Six different positive clones were sequenced; a single clone with complete ORF (pAB007) was selected for sequence analysis and protein expression (Figure 7).

2.22. Heterologous gene expression of linalool synthase

The putative full length *LIS* sequence (including the plastid targeting signal (PTS)) was excised from pAB007 with EcoRI and cloned ‘in frame’ into the pET32a expression vector (Figure 8). The pET expression vector allows for highly efficient heterologous expression of terpene synthases (van Schie et al., 2006) and simultaneously introduces an N-terminal poly-histidine tag that aids subsequent protein purification. The construct was transformed into *E. coli* DH10B competent cells. Eighteen clones were selected and sequenced to ensure directional insertion of the *LIS* gene into the vector backbone. One clone (termed pAB040) (Figure 8), positive for in-frame *LIS* insertion was selected and transformed into the *E. coli* BL21(DE3) which allow for highly efficient expression of recombinant proteins. The same clone was also inserted into the *E. coli* Origami (DE3) host, an *E. coli* K-12 derivative with mutations in the thioredoxin reductase and glutathione reductase genes. These mutations enhance disulfide bond formation in the cytoplasm, and thus facilitate correct protein folding and expression.

2.23. Heterologous protein expression of linalool synthase

For protein expression three clones of pAB040 (termed pAB051, 052 and 053) were selected for the inoculation of 15 ml of LB plus 50 µg/ml of carbenecillin. Cultures were grown at 37°C overnight, diluted 1:40 in 15 ml of LB-carb⁵⁰ and grown to an OD₆₀₀=0.4-0.8. Each culture was split in half, one half was supplemented with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) which induces the expression of the recombinant protein; the other half was left untreated and thus functioned as a control. Induced and non-induced cultures were grown for an additional 12-16 h at room temperature, before cells were harvested by centrifugation at 5000 g at 4°C for 15 min and frozen at -80°C. The total cell protein, soluble and insoluble protein fraction was harvested from this cell preparation as described below.

2.24. Protein purification

2.24.1. Purification of total cell protein

Protein expression was tested by SDS-PAGE analysis of cell extracts followed by staining with Coomassie blue, which in many cases reveals the target protein as a unique band when run adjacent to a non-induced extract. Therefore total cell protein was isolated from induced and non-induced *E. coli* preparations of pAB051, -052 and -053. A 1-ml aliquot of the induced and non-induced culture was taken and centrifuged at 10,000 x g for 1 min. The cell pellet was resuspended in 100 µl of 1 x phosphate-buffered saline (PBS). Subsequently, the sample was supplemented with 100 µl of 2 x Sample Buffer (200 mM DTT, 4% SDS, 100 mM Tris-HCl, pH 6.8, 0.25% bromophenol blue, 20% glycerol) and sonicated with a microtip (Branson Sonifier 450) at the following settings: power level between 2–3, at 20–30% duty for 8–10 bursts. The sample was then heated for 3 min at 80°C to denature the proteins and then stored at -20°C until SDS-PAGE analysis.

2.24.2. Purification of soluble protein

For the isolation of soluble protein, cells were harvested from liquid culture by centrifugation at 6,500 x g for 5 min. the supernatant was decanted and the pellet was drained

of all liquid. Cells were then resuspended in 200 µl of BugBuster solution (Novagen), a protein extraction reagent that causes the gentle disruption of the cell wall of *E. coli*, resulting in the liberation of soluble protein. The cell suspension was incubated on a shaking platform or rotating mixer at a slow setting for 10 min at room temperature. Insoluble cell debris was removed by centrifugation at 16,000 x g for 20 min at 4°C. The pellet was saved for inclusion body purification as described below. The supernatant was mixed with an equal volume of 4 x SDS sample buffer (400 mM DTT, 8% SDS, 200 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 40% glycerol), heated for 3 min at 85°C and stored at -20°C until SDS-PAGE analysis.

2.24.3. Purification of inclusion-bodies

For the isolation of insoluble protein, i.e. purification of inclusion bodies, cells were harvested from liquid culture by centrifugation at 6,500 x g for 5 min. the supernatant was decanted and the pellet was drained of all liquid. Cells were then resuspended in 200 µl of BugBuster solution. The cell suspension was incubated as described above. The pellet was resuspended in 200 µl of BugBuster reagent and shaken vigorously to obtain an even suspension. An additional 6 volumes of 1:10 diluted BugBuster reagent (in deionised water) were added to the suspension and mixed vigorously for 1 min. The suspension was centrifuged at 16,000 x g for 15 min at 4°C to collect the inclusion bodies and the supernatant was removed with a pipette. The inclusion bodies were then resuspended in 500 µl of 1:10 diluted BugBuster, mixed vigorously, and centrifuged as before. This wash step was repeated two more times. The final pellet of purified inclusion bodies was resuspended in 100 µl of 1% SDS and mixed vigorously to solubilise the protein. A 100-µl aliquot of the resulting (insoluble) protein fraction was mixed with three volumes of 4 x SDS sample buffer (see above), heated to 85°C for 3 min and analysed by SDS-PAGE.

2.25. In vitro functional assay

The functionality of the recombinant LIS protein was tested in an *in vitro* assay adapted from assays reported previously (Bohlmann et al., 1997, Faldt et al., 2003, Landmann et al., 2007, Peters et al., 2000). Therefore, protein expression was induced as described above, and cells were harvested from the induced and non-induced cultures by centrifugation at 5000 x g

for 15 min at 4°C. The cell pellet was resuspended in 500 µl of assay buffer (25 mM Tris-HCL pH 7, 5% glycerol, 1 mM DTT, 1 mM MnCl₂, 1 mg/ml of BSA) and disrupted by sonication using a microtip at 15% output at three 30 sec bursts. Cell debris was removed from the sample by centrifugation at 10,000 x g at 4°C for 20 min. The clean supernatant was transferred to a glass vial and supplemented with 50 µM geranyl diphosphate (GPP), mixed and overlaid with 500 µl of diethyl-ether in a sealed tube. The solution was incubated at 30°C for 1 h to allow the recombinant protein to react with the substrate (GPP). The reaction was terminated by vigorous mixing and samples were centrifuged to separate phases. One hundred nanograms of menthol was added as internal standard, before the upper solvent phase was collected. The extraction was repeated once with 500 µl of diethyl-ether. Extracts were pooled and 2 µl were analyzed by GC/MS in split-less mode.

Host	Supplier	Genotype
<i>Escherichia coli</i> (<i>E.coli</i>) Top10	Invitrogen, Canada	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>ara</i> Δ 139 Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^R) <i>endA1</i> <i>nupG</i>
<i>E. coli</i> DH5 α	n/a	F ⁻ , ϕ 80 <i>dlacZ</i> Δ M15, Δ (<i>lacZYA-argF</i>)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk ⁻ , mk ⁺), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>
<i>E. coli</i> DH10B	n/a	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>ara</i> Δ 139 Δ (<i>ara, leu</i>)7697 <i>galU</i> <i>galK</i> λ^- <i>rpsL</i> (Str ^R) <i>nupG</i>
<i>E. coli</i> BL21 (DE3)	Novagen, Canada	F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3)
<i>E. coli</i> Origami (DE3)	Novagen, Canada	F ⁻ <i>ompT</i> <i>hsdS_B</i> (r _B ⁻ m _B ⁻) <i>gal dcm lacY1 ahpC</i> (DE3) <i>gor522:: Tn10 trxB</i> (Kan ^R , Tet ^R)

Table 2: Supplier and genotype of bacterial hosts











Developmental stages of <i>L. x intermedia</i> cv. Grosso									
Flower							Leaf		
Bud I	Bud II	Bud III	Anthesis	30%	70%	Bloomed out	Young	Inter- mediate	Old
≤1 cm	1-2 cm, green	3-4 cm, violet	5-8 cm, violet, unopened flowers	30% of flowers on spike opened	70% of flowers on spike opened	All flowers senescent	2 cm	4.5 cm, green	5 cm, blue green
									

Table 3: Developmental stages of *Lavandula x intermedia* cv. Grosso based on flower/leaf size and colour











Developmental stages of <i>L. angustifolia</i> cv. Munstead									
Flower							Leaf		
Bud I	Bud II	Bud III	Anthesis	30%	70%	Bloomed out	Young	Inter-mediate	Old
≤1 cm	1-2 cm, green	3-4 cm, violet	4-5 cm, violet, unopened flowers	30% of flowers on spike opened	70% of flowers on spike opened	All flowers senescent	2 cm	3 cm, green	4 cm, blue green
									

Table 4: Developmental stages of *Lavandula angustifolia* cv. Munstead based on flower/leaf size and colour.

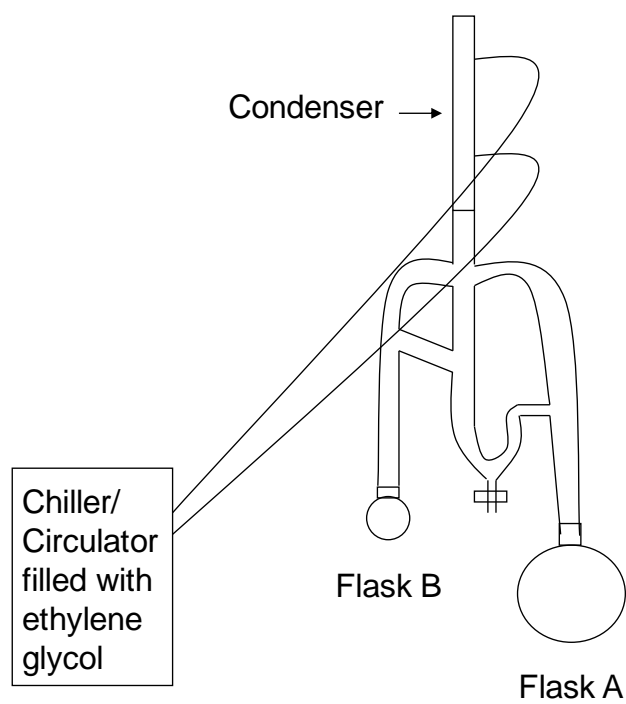


Figure 6: Likens-Nickerson apparatus equipped with a standard condenser used for steam distillation solvent extraction.

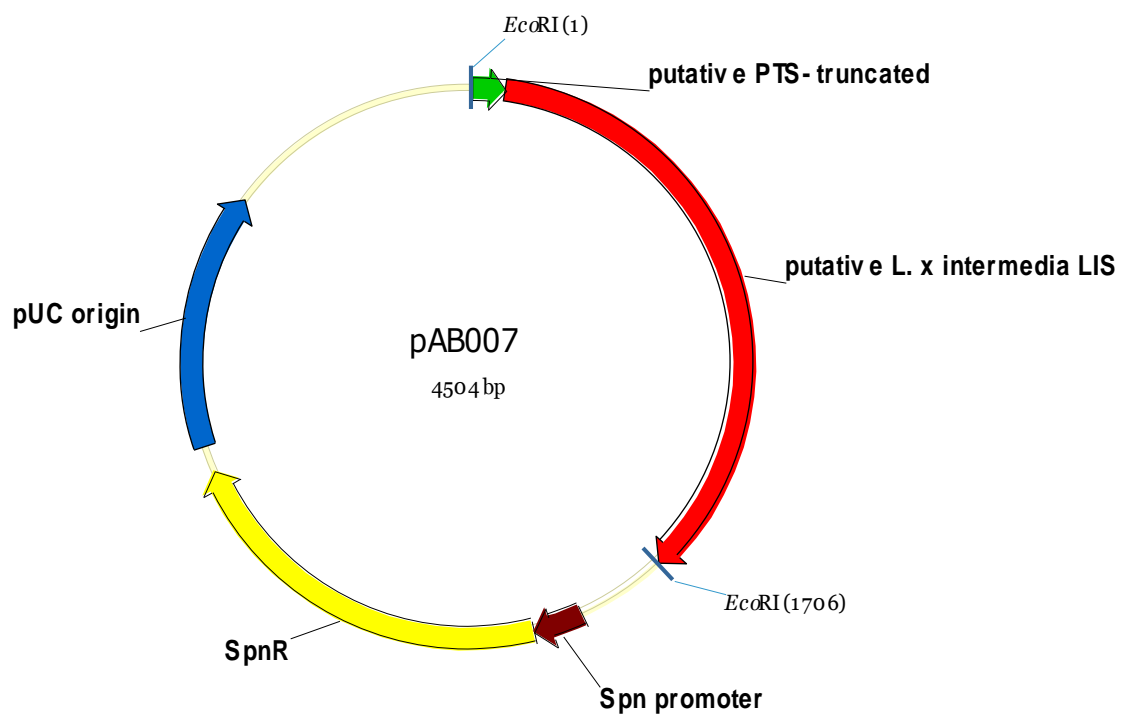


Figure 7: Vector map of pAB007. pAB007 was generated by cloning the *Lxi-LIS* PCR amplification product into pCR®8/GW/TOPO. SpnR: spectinomycin resistance, Spn: spectinomycin, pUC origin: *E. coli* plasmid cloning vector origin of replication, *EcoRI*: Restriction site, *E. coli* RI, PTS: plastid targeting signal.

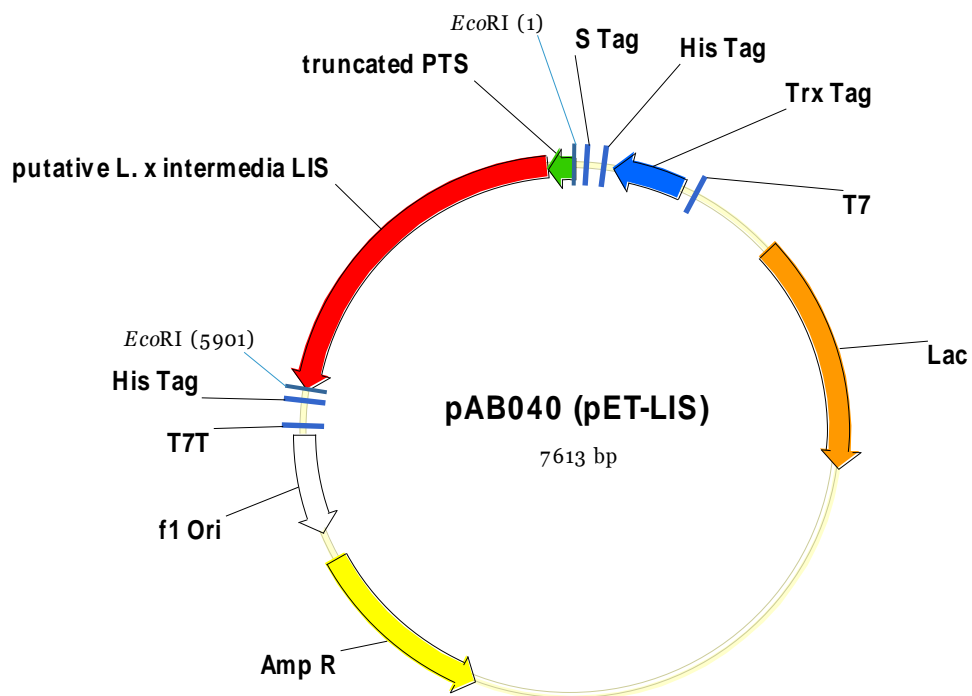


Figure 8: Vector map for pAB040. pAB040 is a vector for the heterologous expression of a putative *Lxi-LIS* in *E.coli*. The vector was created by cloning the *Eco*RI excision product from pAB007 into pET32a. T7: T7 RNA polymerase promoter; Trx Tag: thioredoxin tag; His tag: histidine tag, S tag: S-peptide tag; *Eco*RI: Restriction site, *E.coli* RI; T7T: T7 terminator, f1 ori: filamentous bacteriophage f1 origin of replication; Amp R: ampicillin resistance, Lac: lactose operon repressor; PTS: plastid targeting signal.

3. RESULTS

3.1. Analysis of essential oil from different lavender varieties

The lavender species *L. angustifolia* and *L. x intermedia* are comprised of 50-plus cultivars, each equipped with specialized characteristics that allow their cultivation in distinct climates and confer each variety with a characteristic monoterpene profile. I measured the relative abundance of four major monoterpenes (borneol, camphor, limonene, linalool) in flowers of eleven commercially important lavender cultivars (*L. angustifolia* cvs. Royal Velvet, Royal Purple, Bowles, Munstead, Lavender Lady, Mailette, Hidcote, Premier and *L. x intermedia* cvs. Grosso, Super and Hidcote Giant) and compared the abundance of these compounds between *L. angustifolia* and *L. x intermedia*.

Linalool levels in *L. angustifolia* and *L. x intermedia* cultivars ranged from 12.4% to 34% (Table 5 and 6), with no significant difference between the two species (p-value 0.863, Table 7). Limonene abundance ranged from 2.8% and 6.1% in *L. angustifolia* cultivars (Table 5) and from 2.5% to 4.9% in *L. x intermedia* cultivars (Table 6). Statistical analysis showed that limonene percentages in *L. angustifolia* were significantly higher than in *L. x intermedia* cultivars (p-value <0.001, Table 7). Camphor as well as borneol were detected in traces in *L. angustifolia* varieties (Table 5), but accumulated to 5.1-13.1% (camphor) and 3.7-7.4% (borneol) in *L. x intermedia* cultivars (Table 6) a difference that was statistically significant (p-value <0.001, Table 7). The total amount of essential oil ranged between 37.8 mg/gfWT to 99 mg/gfWT for all *L. angustifolia* cultivars except Mailette which generated the highest oil yield for this species at 91.8- 143.3 mg/gfWT (Tables 5 and 6). In *L. x intermedia* cultivars, total oil accumulated to 116.8-202.8 mg/gfWT and was thus significantly higher (p-value <0.001, Table 7) than for *L. angustifolia* lavenders. In comparison to ISO standards, Okanagan grown lavender displayed a slightly different aroma profile. Borneol levels in *L. x intermedia* cultivars were generally higher than previously reported (ISO 8902, 2002), as were camphor levels in the *L. x intermedia* cultivars Hidcote Giant and Grosso (Table 6). Limonene abundances were higher than the ISO standard (ISO 3515, 1897) in all *L. angustifolia* cultivars tested, while linalool amounts were lower than standardized in all *L. angustifolia* and *L. x intermedia*

cultivars except for the *L. angustifolia* cvs. Munstead, Royal Velvet and Mailette (Tables 5 and 6).

3.2. Monoterpene abundance during lavender development

Recent studies have indicated that monoterpene abundance is subject to developmental regulation (Turner et al., 1999, Turner et al., 2000b, Dudareva et al., 2003). To test this hypothesis in lavender, I measured the amount of borneol, camphor, limonene, and linalool throughout the ontogeny of flowers and leaves. To account for potential species-specific effects, monoterpene abundance was measured throughout the development of two representative cultivars, namely *L. angustifolia* cv. Munstead and *L. x intermedia* cv. Grosso. Monoterpene abundance was measured as a proportion of the total essential oil extracted (relative abundance) to highlight the relationship between all monoterpenes contained in the essential oil, as well as the absolute content (absolute abundance) to allow inferences about the biosynthetic rate of the selected monoterpenes.

3.2.1. Relative abundance of monoterpenes in Grosso and Munstead flowers

Monoterpene abundance changed drastically throughout the ontogeny of both lavender species. One of the predominant developmental trends in flower tissue was the increase in linalool abundance. While both Grosso and Munstead flower buds (Bud I) contained little linalool (4.6% and 1.9%, respectively), linalool abundance increased gradually over time and peaked when approximately 70% of the flower spike was in bloom (Grosso 30.4%, Munstead 30%) (Table 8).

Camphor levels followed a reciprocal trend in Grosso flowers. Camphor accumulated to 18.9% in youngest buds and decreased to 10.7% at the 30% stage. For the remainder of the flower life, camphor levels remained constant at ~10% (Table 8). Borneol, the suspected camphor precursor (Croteau and Karp, 1976, Croteau and Karp, 1979a, Croteau and Karp, 1979b, Dudareva et al., 2003) amounted to 3.6% in Grosso buds; a level that was maintained throughout Grosso flower development with only a slight increase to 4% as the flower reached maturity (Table 8). Camphor was practically absent in Munstead flowers, and borneol remained low, fluctuating between 0.5% and 1.8% (Table 8). Limonene, a likely precursor to

various oxygenated monoterpenes (Karp et al., 1990, Bouwmeester et al., 1998) accumulated to 4.8% in Munstead and 3.2% in Grosso buds, and decreased slightly (to 1.7% and 2.6%, respectively) as the flowers matured (Table 8).

3.2.2. Monoterpene abundance in lavender leaves

The essential oils obtained from Grosso and Munstead leaves were mainly comprised of borneol and camphor. In contrast to flowers, borneol and camphor increased with the age of the leaves. Borneol increased from 6.5% to 9.3% in Grosso, and from 2.7% to 38.6% in Munstead leaves while camphor levels increased from 16.6% to 56.1% in Grosso and 0.5% to 12.1% in Munstead leaves (Table 8). It is noteworthy, that the course of camphor and borneol accumulation in Grosso was reciprocal to their accumulation in Munstead: in Grosso leaves, camphor content was consistently higher than borneol content, while borneol was the predominant monoterpene in Munstead leaves (Table 8).

The leaves of either species contained a substantially lower amount of linalool than flowers and linalool abundance decreased with leaf age. Linalool was detected in minute quantities (1.1% in Grosso, 0.1% in Munstead) in young leaves, was found only in traces in intermediate aged leaves and was practically undetectable in old leaves (Table 8). Similarly, limonene abundance decreased as the leaf matured: limonene abundance was highest in young leaves (1.9% in Grosso, 6.6% in Munstead) but decreased to amounts below the detection limit (Grosso) or traces (0.5% in Munstead) in old leaves (Table 8).

3.2.3. Total oil yield of Grosso and Munstead tissue

As demonstrated in Table 8, the amount of total oil increased gradually over the course of Grosso flower development. The total oil concentration reached maximal levels (32.5 mg/gfWT) at the 30% stage, and decreased slightly thereafter. In contrast, total essential oil yield during Munstead flowers ontogeny reached an early maximum of 16.3 mg/gfWT in green buds (bud II) and remained between 13.9-15.2 mg/gfWT throughout the remainder of flower maturation (Table 8).

The amount of total oil in leaves was negligible compared to the total oil amount in flowers: in old and intermediate leaves total oil accumulated to a maximum of 2.9 mg/gfWT in Munstead and 5.5 mg/gfWT in Grosso (Table 8). Interestingly, the trend of total oil

accumulation in leaves was opposite to the trend in flowers. In leaves total oil decreased with age, while total oil in flowers increased with maturation (Table 8).

3.2.4. Absolute abundance of monoterpenes in Grosso and Munstead

Similar to the pattern observed for the relative abundances, linalool was the predominant monoterpene in both Munstead and Grosso flowers (Table 9). Its concentration rose continuously from youngest buds to mature flowers, and was effectively absent from vegetative tissue (Table 9). The concentrations of limonene and borneol followed similar trends. Both compounds decreased continuously during Munstead flower development (Table 9). In contrast, limonene and borneol amounts during Grosso development followed a bell shaped distribution with peak concentrations when 30% of the flower was in bloom (Table 9). Camphor was detected in trace amounts in Munstead flowers, in contrast to camphor levels in Grosso flowers which- averaging between 50.3-102.3 ng/gfWT at all stages of flower development- were comparably high (Table 9).

In Munstead and Grosso leaves, linalool and limonene concentrations were low (continuously below 10 ng/gfWT for linalool and below 37.8 ng/gfWT for limonene), and both monoterpenes decreased as the leaf matured (Table 9). In Munstead leaves, camphor concentrations increased as the tissue aged but were always lower than borneol concentrations. In contrast, borneol amounts fluctuated between 26.4 ng/gfWT and 42.4 ng/gfWT (Table 9). In Grosso leaves, borneol decreased gradually as the leaf matured and remained consistently below camphor amounts. Camphor amounts on the other hand did correlate less with the developmental state of the leaf, and fluctuated between 10.9 ng/gfWT and 37.4 ng/gfWT (Table 9).

3.3. Isolation of a putative linalool synthase from *L. x intermedia*

Using Reverse Transcriptase PCR (RT-PCR) and primers against a published *L. angustifolia* LIS, I isolated a 1700 bp sequence from *L. x intermedia* flowers, a continuous open reading frame (ORF) of 1639 bp. The 1639 bp ORF coded for a protein with 593 amino acids (aa) (Figure 9), and a predicted molecular weight of 69 kDa. Blast search as well as sequence alignment of this sequence revealed highest sequence identity (87.8%) and amino

acid identity (90.6%) to *L. angustifolia* LIS (Figure 9) suggesting that the isolated sequence represents a *L. x intermedia* LIS homolog (*Lxi-LIS*). Four monoterpene synthase characteristic motifs, RRx8WD, LQLYEASFLL, DDxxD and (N,D)D(L,I,V)X(S,T)XXXE, were completely conserved in the *L. x intermedia* sequence (Figure 9). Similar to other published LIS sequences, the ORF contains a 53 aa N-terminal extension upstream of the RRx8WD motif. The first 25 aa of this extension are highly similar to a targeting signal found in *L. angustifolia* LIS, while the remaining 28 aa show weak similarity to the N-terminus of *L. latifolia* LIS (Figure 9). This N-terminal sequence contained comparatively many serine and alanine residues and few acidic amino acids, a feature that is often found in signal peptides, which target proteins to plastids where they are processed to their active mature forms by truncation of the N-terminal peptides (Keegstra et al., 1989, von Heijne et al., 1989). It is likely that the N-terminal extension of this newly isolated gene also functions as a signaling sequence and is therefore referred to as a plastid targeting signal (PTS) from here on.

3.4. Heterologous expression and functional analysis of a putative *L. x intermedia* LIS

The sequence of the isolated ORF, including the sequence encoding for a 25 aa portion of the putative signal peptide was cloned and expressed in bacteria. The protein expression levels of three identical expression clones (pAB051, pAB052 and pAB053) were compared to a control that did not contain the isolated sequence (empty pET32a), and to protein preparations of pAB051 and pAB053 which did not express the protein (non-induced samples). As shown by SDS-PAGE, two clones (pAB051 and pAB053) expressed high levels of a 69 kD protein, which was absent from the non-induced controls (Figure 10). However, the major portion of the recombinant protein was trapped in insoluble inclusion bodies, and only a minute sub-fraction was soluble (Figure 10). Even the transfer of the expression vector into *E. coli* Origami cells, an exclusive host strain that promotes disulfide bond formation and thus increases protein solubility (Prinz et al., 1997) failed to improve protein solubility (data not shown).

The soluble fraction of the recombinant protein was tested for monoterpene synthase activity, i.e. its ability to convert geranyl diphosphate to linalool *in vitro*. To account for the low solubility, the *in vitro* assay was scaled up in an attempt to increase the recovery of soluble

Lxi-LIS in the assay. Table 10 summarizes the product profiles of the two *Lxi-LIS*-expression clones (pAB051, pAB053) in comparison to three negative controls, i.e. protein preparations of non-induced pAB051 and pAB053, and an induced sample of the empty pET32a expression vector. The overall extraction efficiency was estimated based on the recovery of an internal standard (menthol) added to the assay after incubation and prior to extraction. In general, 60-70% of the internal standard was recovered during two rounds of ether extraction (Table 10). Extractions of pET32a were particularly efficient with 99% recovery, while the recovery rate for the pAB051-induced sample was only 10%, indicating a failure in the extraction procedure (Table 10). Apart from menthol, only myrcene and linalool could be detected in the ether extracts. Myrcene concentration fluctuated between 0.4 ng/μl and 4 ng/μl, with higher concentrations in non-induced controls. Linalool was produced at low levels in the pAB053-induced sample, but was also found in the pAB051-non-induced control as well as the pET32a control (Table 10). To account for differences in recovery across samples, the data set was mathematically normalized to 100% recovery. Normalization emphasized slightly elevated linalool amounts in the pAB053 induced sample, as compared to the controls (Table 10). This suggests *de novo* production of linalool, and thus indicates that the recombinant protein comprises *LIS* functionality. However, the measured linalool amounts were below the limit of quantitation for linalool (1 ng/μl) and thus remain questionable.

3.5. Correlation of linalool abundance and *LIS* gene expression

The abundances of *LIS* from Munstead as well as the abundance of the sequence isolated from *L. x intermedia* (putative *LIS* homologue, *Lxi-LIS*) were compared to linalool concentrations in the respective cultivar throughout development. Transcription analysis was limited to flower tissue, since no *LIS* could be detected in leaves.

The quantitative changes of *LIS* and *Lxi-LIS* transcripts during Munstead and Grosso flower development, in comparison to linalool abundance are depicted in Figures 11 and 12, respectively. *LIS* and *Lxi-LIS* expression closely paralleled linalool concentration throughout Munstead and Grosso development (Figures 11 and 12). In both cultivars, linalool as well *LIS* / *Lxi-LIS* transcript numbers increased as the flower matured. In Munstead flowers *LIS* transcription decreased in the final stages of ontogeny (i.e. bloomed out flowers), while the abundance of linalool remained high (Figure 11). In contrast, linalool levels and *Lxi-LIS*

abundance decreased in tandem in maturing Grosso flowers (Figure 12). A correlation coefficient of 0.58 (Munstead) and 0.81 (Grosso) indicated the correlation between transcript abundance and product concentration.

Monoterpene content (%) in <i>Lavandula angustifolia</i> cultivars									
Compound	Royal Velvet	Royal Purple	Munstead	Lavender Lady	Bowles	Premier	Hidcote	Maillette	ISO 3515 1987
Borneol	1.1 (1-1.4)	1.1 (1-1.2)	0.7 (0.6-0.7)	1.2 (1.2-1.3)	0.3 (0.2-0.5)	0.9 (0.8-1.1)	1.8 (1.6-2.1)	2.3 (2.3-2.4)	n/a
Camphor	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.4	0-0.5
Limonene	5.7 (5.5-5.9)	5.2 (4.9-5.4)	5.8 (5.6-6.0)	5.7 (5.6-5.9)	3.1 (2.8-3.4)	5.8 (5.6-6.1)	5.9 (5.6-6.0)	5.7 (5.6-5.8)	0-0.5
Linalool	29.4 (27.5-30.6)	18.2 (17.2-19.6)	26.9 (25.5-29.6)	22.6 (21.3-23.3)	21.2 (18.9-23.1)	13.8 (12.4-15.3)	15.1 (13.8-16.1)	33.8 (33.6-34)	25-38
Estimated total oil (mg/gfWT)	71.8 (63.2-78.1)	55.2 (47.6-59.5)	62.0 (50.3-68.6)	68.1 (50.2-99.9)	43.3 (37.8-53.5)	57.1 (53-60.5)	52.2 (44.9-57.8)	117.5 (91.8-143.3)	n/a

Table 5: Monoterpene content (%) and estimated amount of total oil (mg/g fresh weight) extracted from flowers of *Lavandula angustifolia* cultivars grown in the Okanagan, 2007. Relative amounts of selected monoterpenes were measured in essential oil extracts by GC/MS. Monoterpene content is reported as the average of two extractions times two analytical replicas of each pooled tissue sample. The measurement ranges, i.e. minimum and maximum measures, are reported in brackets. n.d.: none detected. n/a: non-available. mg/gfWT: mg per gram fresh weight. ISO 3515 1987: International Standardization Organization, file 3515, year 1987.

Monoterpene content (%) in <i>L. x intermedia</i>				
Compound	Hidcote Giant	Grosso	Super	ISO 8902 2002
Borneol	4.8 (4.4-5.1)	3.7 (3.4-4.1)	7.4 (7.3-7.4)	1.5-3
Camphor	21.5 (11.3-13.1)	10.2 (9.7-10.7)	5.2 (5.1-5.4)	6-8
Limonene	2.5 (2.5-2.6)	4.0 (4.0-4.1)	4.9 (4.8-4.9)	n/a
Linalool	16.9 (16.3-17.6)	23.2 (22.9-23.5)	21.5 (20.5-22.5)	25-35
Estimated total oil (mg/gfWT)	162.7 (147.3-180.8)	182.9 (168.1-202.8)	126.5 (116.8-136.2)	n/a

Table 6: Monoterpene content (%) and estimated amount of total oil (mg/g fresh weight) extracted from flowers of *Lavandula x intermedia* cultivars grown in the Okanagan, 2007. Relative amounts of selected monoterpenes were measured in essential oil extracts by GC/MS. Monoterpene content is reported as the average of two extractions times two analytical replicas of each pooled tissue sample. The measurement ranges, i.e. minimum and maximum measures, are reported in brackets. n.d.: none detected. n/a: non-available. mg/gfWT: mg per gram fresh weight. ISO 8902 2002: International Standardization Organization, file 8902, year 2002.

	Borneol	Camphor	Limonene	Linalool	Total oil
p-value	<0.001	<0.001	<0.001	0.863	<0.001

Table 7: Comparison of the monoterpene percentage in *L. angustifolia* and *L. x intermedia* cultivars. The average percentages (averages of two extractions times two analytical replicas of each pooled tissue sample) of borneol, camphor, linalool and limonene in eight *L. angustifolia* cultivars (n=8) were compared to the average percentage of these monoterpenes in three *L. x intermedia* cultivars (n=3) using the non-parametric Kruskal-Wallis test. P-values indicate the probability that the percentage of the indicated monoterpene is equal between *L. angustifolia* and *L. x intermedia* cultivars. P-values below $\alpha=0.05$ are significant.

Table 8: Monoterpene content (%) during lavender development. Mean relative abundance of borneol, camphor, linalool, limonene and total oil in *L. x intermedia* cv. Grosso (upper panel) and *L. angustifolia* cv. Munstead (lower panel), as measured during the 2007 flowering season. The range of measurements (minimum – maximum) over two extraction and three analytical replications (six samples) is given in brackets.

Monoterpene content (%) in <i>L. x intermedia</i> cv. Grosso and <i>L. angustifolia</i> cv. Munstead during development											
		Flower							Leaf		
		Bud I	Bud II	Bud III	Anthesis	30%	70%	Bloomed out	Young	Inter-mediate	Old
<i>L. x intermedia</i> cv. Grosso	Borneol	3.6 (3.1-4.1)	3.5 (3-4.9)	3.0 (2.6-3.5)	2.9 (2.3-3.9)	4.2 (3.4-5.3)	4.5 (3.7-5)	4.0 (3.4-5.1)	6.5 (4.5-9.4)	9.3 (2.9-15.3)	9.3 (7.6-11.7)
	Camphor	18.9 (15.2-22.2)	17.4 (15.4-21.1)	13.2 (10.6-15.3)	10.6 (9.3-13.6)	10.7 (8.9-13.1)	11.5 (10.7-12.5)	10.7 (9.6-12.6)	16.6 (10.2-32.6)	23.6 (6-41.7)	56.1 (46.9-65.0)
	Limonene	3.2 (2.6-4.2)	3.2 (2.6-4.1)	2.8 (2.1-3.8)	2.9 (1.9-4)	2.4 (1.8-4.5)	2.4 (1.7-4.2)	2.6 (1.9-4.2)	1.9 (0-3.3)	0.1 (0-2.8)	n.d.
	Linalool	4.6 (3.6-5.3)	9.8 (7.3-10.9)	15.1 (11.1-17.2)	20.3 (15.1-23.5)	28.1 (21.3-33.3)	30.4 (20.6-37.1)	29.8 (21.7-37.6)	1.1 (0-1.7)	0.3 (0-1.7)	n.d.
	Estimated total oil (mg/gFWT)	12.2 (11.6-13)	12.1 (11-13.4)	14.7 (12.4-20.4)	15.7 (14.8-16.8)	32.5 (27.2-35.5)	17.9 (15.7-20.3)	23.5 (20.8-25.4)	5.5 (4.3-8.3)	1.9 (0.9-4)	1.3 (0.9-1.6)
<i>L. angustifolia</i> cv. Munstead	Borneol	1.0 (0-1.9)	0.5 (0-1.3)	1.2 (0.9-1.5)	1.8 (1-2.3)	0.6 (0-1.1)	0.9 (0.8-1)	0.7 (0.6-0.8)	2.7 (0-8.2)	23.7 (26.7-30.3)	38.6 (29.4-43.5)
	Camphor	n.d.	n.d.	n.d.	(0-0.3)	n.d.	n.d.	(0-0.2)	0.5 (0.4-0.6)	8.2 (7.4-8.9)	12.1 (10-13.8)
	Limonene	4.8 (0-7.5)	2.5 (0-6.8)	4.2 (3.8-4.9)	3.0 (1.9-3.7)	2.6 (1.9-3.1)	2.3 (2.1-2.5)	1.7 (0.9-2.1)	6.5 (5.7-7.4)	1.2 (0-2.4)	0.4 (0-1.1)
	Linalool	1.9 (1.3-3)	4.5 (0-10.7)	17.0 (15.4-19.8)	16.8 (10.4-20)	17.5 (8.9-24.7)	30.0 (28.2-31.6)	27.7 (4.7-40.8)	0.1 (0-0.2)	n.d.	0.2 (0-0.6)
	Estimated total oil (mg/gFWT)	15 (12.5-21.3)	16.3 (12.8-25.7)	14.4 (12.7-15.7)	13.9 (9.7-20.8)	17.5 (12.9-20.6)	15.2 (13.8-16.7)	15.3 (12.9-18.7)	2.9 (1.9-3.8)	0.8 (0.8-0.9)	0.7 (0.6-0.8)

Table 9: Monoterpene content (ng/gfWT) in *L. x intermedia* cv. Grosso and *L. angustifolia* cv. Munstead during development. Absolute concentration of borneol, camphor, linalool, limonene and total oil was measured in Grosso and Munstead during the 2007 flowering season. The range of measurements over two extraction and three analytical replications (six samples) is given in brackets.

Monoterpene content (ng/gfWT) in <i>L. x intermedia</i> cv. Grosso and <i>L. angustifolia</i> cv. Munstead during development											
		Flower							Leaf		
		Bud I	Bud II	Bud III	Anthesis	30%	70%	Bloomed out	Young	Inter-mediate	Old
<i>L. x intermedia</i> cv. Grosso	Borneol	12.3 (6.3-21.1)	10.9 (5.6-27.3)	11.9 (5-34.2)	14.4 (0.3-34.9)	35 (1.2-90)	16.5 (5.3-44.7)	22.8 (9-62.9)	13.3 (0.6-23)	4.0 (1.5-8.6)	3.8 (1.7-9.4)
	Camphor	74.8 (43.3-119.6)	63.1 (32.1-141)	59 (24.7-152.4)	58 (0-125.9)	102.3 (2.8-242.6)	50.3 (18.7-124)	73 (33.9-183.1)	37.4 (1.0-65.6)	11 (3.3-30.4)	26.5 (12.5-59.8)
	Limonene	8.3 (3.7-16.5)	8.8 (2.8-29.9)	14 (3.1-48.8)	23 (0-58.9)	46.3 (0.9-140.7)	16.1 (1.8-55)	25.5 (4.8-98.8)	6.5 (0-10.3)	1.5 (0.5-5.4)	2.1 (1.3-3.6)
	Linalool	93.9 (34.5-298)	212.6 (51.6-756.2)	436.8 (74.9-1617.1)	844.5 (0-2204.9)	1699.2 (25.1-5220.5)	756.9 (110.8-2770.1)	1073.8 (211.9-4050.4)	7.4 (0-13.5)	1.0 (0-5.2)	n.d.
<i>L. angustifolia</i> cv. Munstead	Borneol	27.8 (10.2-68.2)	22.5 (18.3-28.2)	13.6 (11.5-15.3)	23.1 (17.1-27.9)	14.7 (6.6-21.3)	9.7 (8.9-10.8)	9.2 (5.5-15.4)	42.4 (33.9-53.1)	26.4 (21.6-32)	31.8 (27-34)
	Camphor	2.1 (0-6.9)	n.d.	0.5 (0-2.8)	2.6 (0-4.6)	2.6 (0-4.6)	2.9 (2.5-3.2)	4.1 (3.4-4.6)	2.8 (2.1-3.5)	8.8 (7.8-9.9)	12.6 (11.6-13.8)
	Limonene	156.9 (84.7-301.3)	134 (111.9-159.4)	65.2 (48.5-81.3)	52.3 (37.4-70.4)	64 (32.6-104.6)	42.6 (38.1-47.7)	35.7 (14.1-61.3)	37.8 (22.2-54.7)	1.5 (0.7-2.6)	1.2 (0.8-1.6)
	Linalool	114.5 (42.7-248)	531.4 (489.1-559.8)	573.5 (453.6-707.6)	639 (452.3-912)	1206.3 (642.5-2033.3)	1148.8 (1068.9-1273.1)	1575.8 (1022.6-2336)	2.3 (2-2.6)	0.8 (0.6-1)	1.4 (0-4.7)

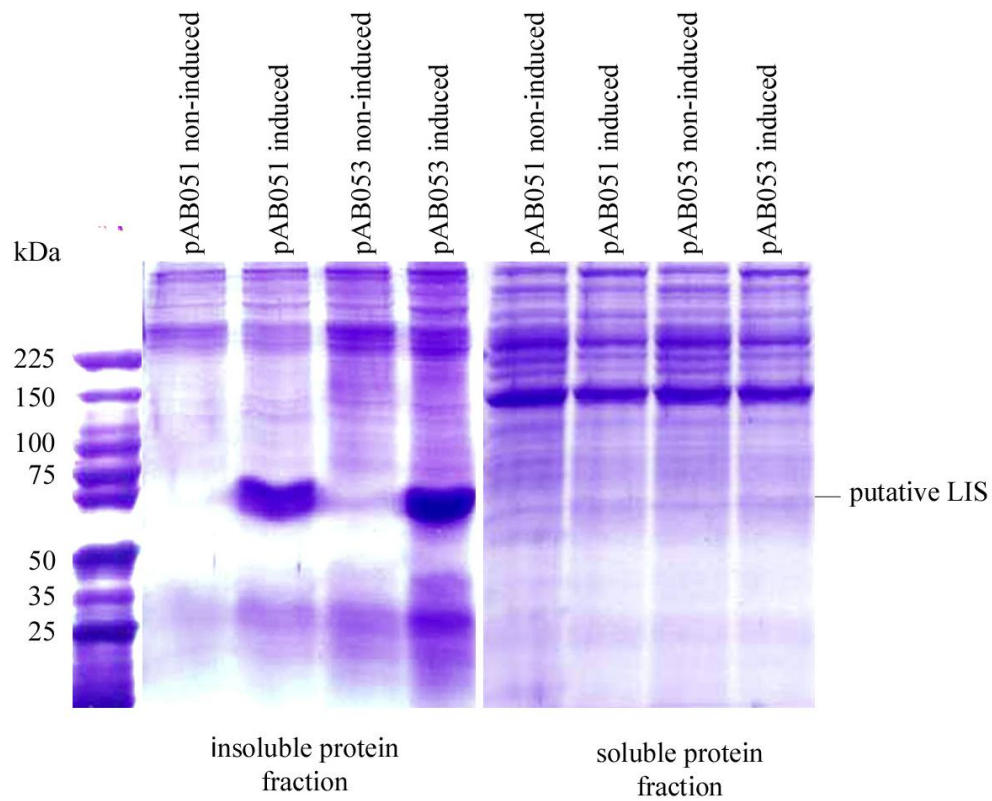


Figure 10: Heterologous expression of putative *L. x intermedia* LIS. The putative LIS was expressed in *E. coli* (clones pAB051 and pAB053); the soluble and insoluble protein fractions were isolated and compared to the protein extract from non-induced, i.e. non-expressing clones.

<i>In vitro</i> functional expression assay of <i>Lxi</i> -LIS						
		pAB051 Induced	pAB051 non-induced	pAB053 induced	pAB053 non-induced	pET32a induced
Monoterpene synthesis	Recovery (%)	9.78	62.89	65.05	69.62	98.86
	Myrcene (ng/μl)	0.43	3.99	0.67	2.25	0.70
	Linalool (ng/μl)	n.d.	0.25	0.14	n.d.	0.13
Normalized Monoterpene synthesis	Recovery (%)	100	100	100	100	100
	Myrcene (ng/μl)	4.42	6.34	1.03	3.23	0.71
	Linalool (ng/μl)	n.d.	0.40	0.21	n.d.	0.13

Table 10: *In vitro* functional expression assay. A heterologously expressed, putative *Lxi*-LIS was tested for its ability to convert GPP to linalool or other monoterpenes. Reaction products were extracted from the *in vitro* assay and analysed by GC/MS. Monoterpene concentrations were measured in a 2 μl extract and compared to background monoterpene levels measured in non-induced intra-clonal (non-induced) or induced inter-clonal (pET32a induced) controls. Extraction recovery was calculated based on the recovery of 100 μg of menthol added to the assay. Normalization adjusted the monoterpene abundance to a hypothetical recovery of 100%.

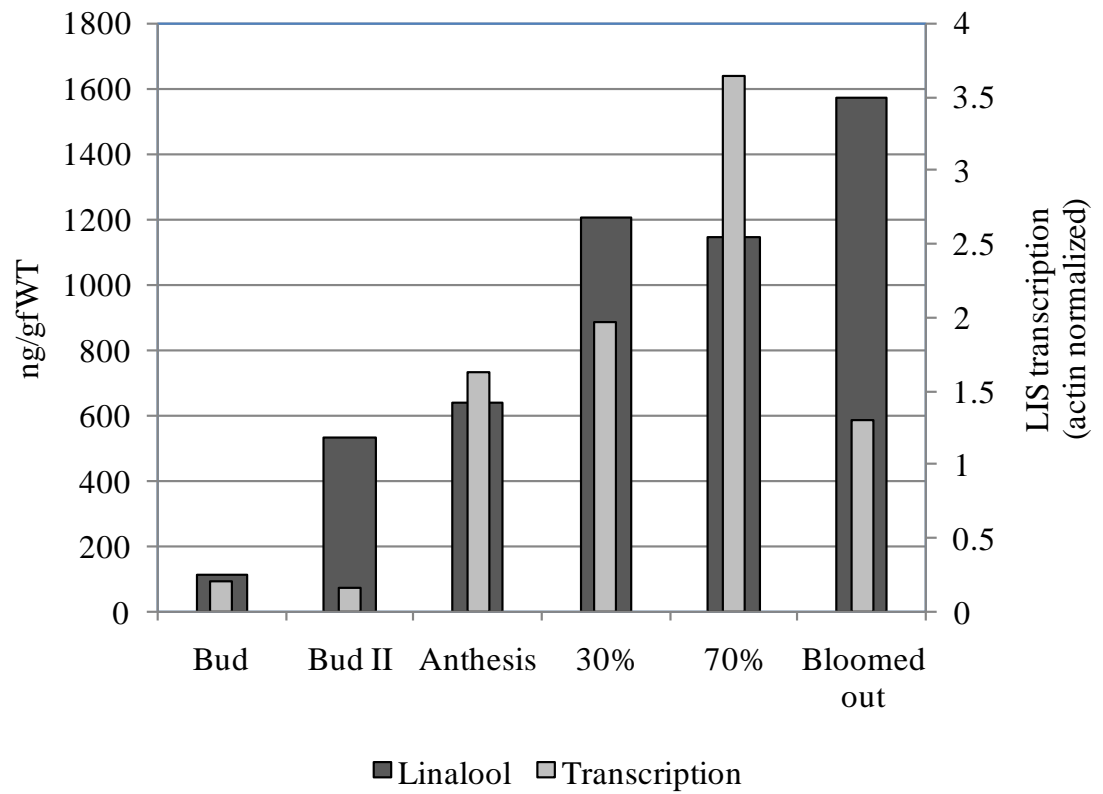


Figure 11: Comparison of linalool concentration and *LIS* transcription during Munstead development. *LIS* transcription levels obtained by Real-Time PCR were normalized to actin transcript levels in the same tissue, and compared to the average linalool abundance (ng/gfWT) measured previously.

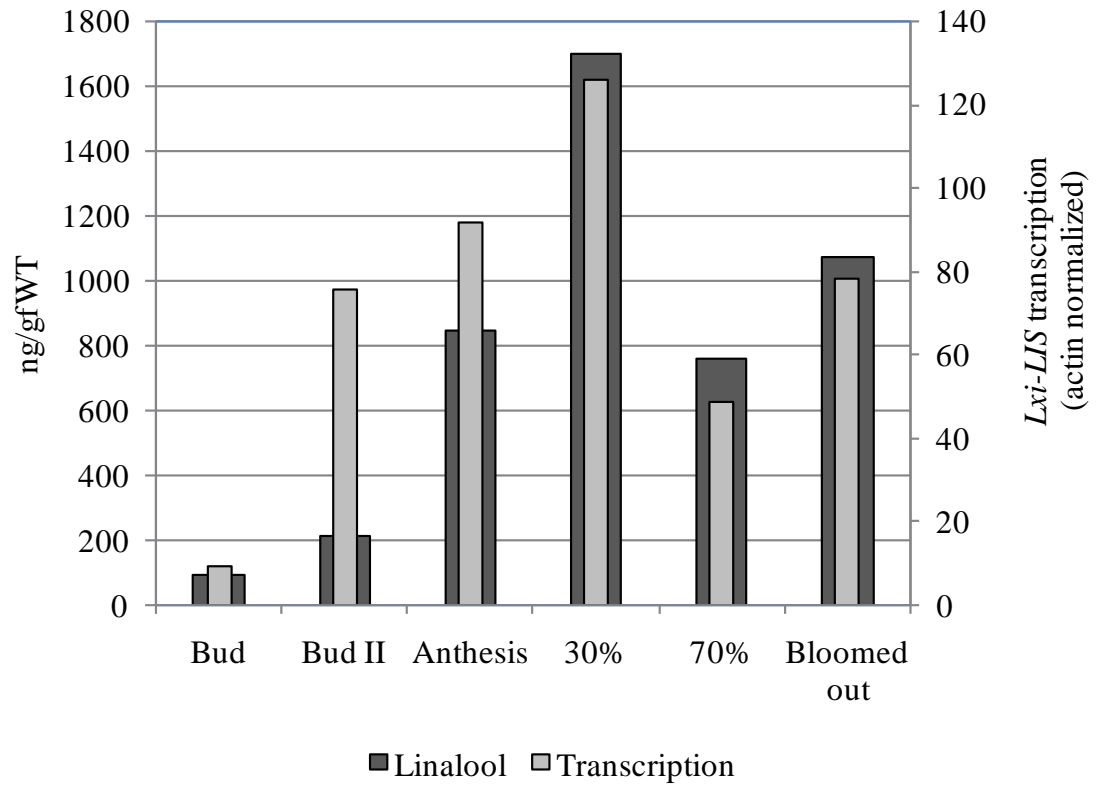


Figure 12: Comparison of linalool concentration and *Lxi-LIS* transcription during Grosso development. *Lxi-LIS* transcription levels obtained by Real-Time PCR were normalized to actin transcript levels in the same tissue, and compared to the average linalool abundance (ng/gfWT) measured previously.

4. DISCUSSION

Lavender essential oil is a complex mixture of monoterpenes (and to a lesser extent sesquiterpenes), which give the plant its characteristic aroma. Linalool is one of the most abundant monoterpenes found in lavender oil and the ratio between linalool and camphor determines oil quality. High linalool concentrations combined with trace amounts of camphor are associated with high quality oil, extracted from *L. angustifolia*. In contrast, elevated camphor quantities characterize low quality oil from *L. x intermedia*. It is widely assumed that the production of linalool and camphor are regulated genetically, and that the difference in camphor levels in *L. angustifolia* and *L. x intermedia* is due to the distinct genetic background of these species. Monoterpene production is also influenced by climate and varies throughout the development of the plant (Clark and Menary, 1980a, Clark and Menary, 1980b, Hussein et al., 1996, Turner et al., 2000a, Turner et al., 2000b, Dudareva et al., 2003, reviewed in Sangwan et al., 2001). This study was designed to determine the abundance of major monoterpenes in lavender cultivars grown under the special climatic conditions in the Okanagan. Under constant climatic conditions, the differences in monoterpene abundance between the lavender species *L. angustifolia* and *L. x intermedia* and throughout tissue development were determined. Measurements of monoterpene abundance were limited to linalool and camphor, as the major quality determining monoterpenes as well as biosynthetically related compounds, i.e. borneol, the putative camphor precursor, and limonene, a precursor for a wide variety of oxygenated monoterpenes (Karp et al., 1990)). Linalool acetate, the product of linalool acetylation and a major monoterpene in most lavenders, was omitted from the analysis, since linalool acetate detection was suboptimal and thus unreliable. Together, the abundances of linalool, camphor, borneol and limonene allowed inferences about the regulation and the biosynthetic pathways of these monoterpenes and thus set the study into a broader, more comprehensive context.

4.1. Monoterpene abundance is species-specific

For the last century, lavender has been mainly cultivated in France, Spain and Britain. French lavender is traditionally considered to produce highest quality oils, as determined by a

high abundance of linalool and low levels of camphor. The Okanagan Valley of British Columbia is a fairly new region for lavender cultivation, characterized by a unique mix of hot, arid summers and cold winters. Given these particular environmental conditions, it seemed likely that lavender grown in the Okanagan would display unique essential oil characteristics. Indeed, phytochemical analysis showed that the abundances of camphor and linalool in Okanagan grown lavender differed slightly from the expected range framed by ISO (*L. angustifolia* ISO 3515, *L. x intermedia* cv. Grosso ISO 8902). Linalool levels were generally slightly lower than the expected range in all cultivars, while camphor abundances were within the expected range in *L. angustifolia* but slightly higher than the standard in *L. x intermedia* cultivars.

Comparison of the monoterpene content of multiple *L. angustifolia* and *L. x intermedia* cultivars at the species level demonstrated that both species accumulated similar amounts of linalool. In contrast, the proportion of camphor as well as the total oil yield was higher in all *L. x intermedia* cultivars, which indicated that camphor abundance and total oil yield were defined by the genetic background of the species and were likely genetically predetermined.

The higher oil yield of *L. x intermedia* cultivars may be a consequence of the higher overall productivity of *L. x intermedia* cultivars. *L. x intermedia*, a *L. angustifolia*-*L. latifolia* hybrid, is highly prolific, in contrast to the smaller growth of *L. angustifolia* plants. This strong and vigorous growth that often exceeds the growth characteristics of the parent species is a well described phenomenon in hybrid species, known as hybrid vigor or heterosis. Heterosis might affect essential oil yield either through a simple increase in tissue mass or through an increase in the biosynthetic capacity of the plant caused by changes in the genetic background of the hybrid. Hybridization usually affects a large number of genes or gene combinations, making it difficult to assign the difference in essential oil yield to a single changed gene. However, it can be speculated that elevated oil levels in *L. x intermedia* are related to an increase in the activity of regulatory enzymes that control the availability of common precursors. For example, up regulation of enzymes that determine the flux through the DXP-or MVA pathway may increase the availability of the monoterpene precursor GPP. Alternatively, elevated oil yield in *L. x intermedia* might be due to an increase in storage capacity, i.e. an increase in the density of essential oil glands. One argument in support of this hypothesis was the higher essential oil yield in young leaves as compared to old leaves, a pattern that closely

resembled essential oil accumulation in mint leaves (*Mentha sp.*) (Turner et al., 2000b). The accumulation of essential oil in young mint leaves was explained by the steady increase of the total number of peltate glands as the season progressed. Leaves produced early in the season (i.e. old leaves at the time of harvest) had fewer glands per leaf than leaves produced later in the season (young leaves at time of harvest), even though the overall size of the leaf blade was comparable (Colson et al., 1993). It is conceivable that essential oil/monoterpene accumulation in lavender is correlated with the population dynamics of peltate oil glands in a similar fashion. However, further analyses are required to measure trichome numbers on lavender tissue and define the effect of trichome density on essential oil yield.

4.2. Camphor abundance as an indication of the camphor biosynthetic pathway

One observation in this study was that borneol and camphor were differentially abundant in *L. x intermedia* and *L. angustifolia* as well as in leaves and flowers. Leaves generally contained these two compounds in abundance, while camphor and borneol were lower in flower tissue. Interestingly, higher camphor abundance in *L. x intermedia* cv. Grosso was generally accompanied by low borneol abundance, while this pattern was reversed in Munstead.

Studies in sage (*Salvia officinalis*) showed that camphor and borneol are biosynthetically related. In sage, camphor is synthesized from geranyl diphosphate in a three step process (Figure 13). After the initial conversion of GPP to bornyl diphosphate (BPP) by bornyl-diphosphate synthase (BPPS), BPP is hydrolysed to borneol by bornyl-diphosphate hydrolase (BPPH). The pathway is completed by a terminal oxidation of borneol to camphor by borneol dehydrogenase (BDH) (Figure 13) (Croteau et al., 1978, Croteau and Karp, 1979a, Croteau and Karp, 1979b, Dehal and Croteau, 1987, Wise et al., 1998). In theory, the presence or absence of these three enzymes determines the abundance of the intermediates and the end product. When all three enzymes are expressed, GPP will be converted mainly into camphor, which will accumulate as the end product. If either BPPH or BDH is inactive or absent, bornyl-diphosphate or borneol will accumulate instead while camphor abundance will remain low.

Assuming an analogous pathway in lavender, the observed distribution of borneol and camphor in Munstead and Grosso tissue was likely caused by the differential expression or activity of BPPH and BDH in these two species. In Grosso, BPPH and BDH might both have

been highly active, catalyzing the production of borneol followed by its conversion into camphor. Thus, camphor accumulated and camphor levels were consistently higher than borneol concentrations in *L. x intermedia*. In Munstead however, camphor was effectively absent from flowers, and remained low in leaves as compared to Grosso leaves, while borneol exceeded camphor concentrations in both tissues. This implies that although both BPPH and BDH were expressed, BDH expression/activity in Munstead was lower than in Grosso causing the accumulation of borneol rather than camphor.

4.3. An ecological view of the tissue-specific expression of monoterpenes

The analysis of lavender essential oil demonstrated that the abundances of linalool and camphor were significantly different between vegetative and reproductive tissues. Camphor abundance in leaves was much higher than in flowers, irrespective of lavender species, while linalool concentrations were significantly higher in flowers than in leaves. This tissue specific abundance may be explained through the distinct ecological function of linalool and camphor in vegetative and reproductive tissue. In vegetative tissues, monoterpenes act in direct and indirect defense against herbivores and as a protection against plant pathogens (reviewed in Dudareva and Pichersky, 2000). Camphor is known to encompass insecticidal and anti-microbial activity, and may thus play a role in the defense of lavender pathogens, primarily affecting vegetative tissue. In flowers on the other hand, monoterpenes likely serve as volatile cues to attract insects and other pollinators (Knudsen et al., 1993, Dobson, 1994, Dudareva and Pichersky, 2000). Linalool, which is the predominant monoterpene of lavender flowers, is present in the floral fragrance of diverse plant families and is attractive to a broad spectrum of pollinators. It has been shown that linalool emission is often synchronized with pollinator activity. For instance, emission of linalool from *Clarkia breweri* (Gray) Greene (*Onagraceae*) flowers evolved to suit pollination by nocturnal moths, while linalool emission in its evolutionary progenitor sibling species *C. concinna* (Fischer & Meyer) Greene, matched the diurnal activity pattern of their pollinating bees (MacSwain et al., 1973, Raguso and Pichersky, 1995, Raguso and Pichersky, 1999). Interestingly, studies also showed that linalool emission and flower scent were markedly reduced soon after pollination (Dudareva and Pichersky, 2000), an observation that might reason the slight decrease in the average linalool abundance in mature lavender flowers. The practical absence of linalool from leaves is explained by the

hypothesis that fragrance compounds active in pollinator attraction are emitted exclusively from flowers, since volatiles emitted from both leaves and flowers would be unreliable to orientate foraging pollinators to the flowers at close range (Pellmyr et al., 1987). The cause of the tissue-specific distribution of camphor and linalool is so far speculative. Nevertheless, a better understanding of the (tissue-specific) distribution of monoterpenes that deter predators or allure pollinators is of particular interest for agriculture. For instance, monoterpenes functioning as natural protectants may be utilized for ecological pest control and thus reduce the need for chemical pesticides. Natural pollinator attractants on the other hand may enhance the success rate of fertilization and improve both crop yield and quality.

4.4. Isolation of a putative linalool synthase from *L. x intermedia*

Linalool synthase (*LIS*), the monoterpene synthase catalyzing the production of linalool, has been isolated from a number of different plants including the two *L. x intermedia* parental species *L. angustifolia* and *L. latifolia*. During the course of this study a cDNA clone from *L. x intermedia* (cv. Grosso) was isolated, which showed highest similarity to *L. angustifolia* *LIS*. The similarity between the *L. angustifolia* *LIS* and the *L. x. intermedia* clone suggested that this sequence functions as a *LIS* homolog, and was therefore termed *Lxi-LIS*. Sequence comparison of the newly isolated *Lxi-LIS* sequence to other monoterpene synthases, placed the former gene into the TPS-b class of monoterpene synthases (Figure 14), together with other *LIS* representatives of the *Lamiaceae* family. Amino acid analysis showed that this putative *LIS* encompassed an N-terminal extension with comparatively many serine and alanine residues and few acidic amino acids. This feature is often found in signal peptides, which direct proteins to plastids where they are processed to their active mature forms by truncation of the N-terminal peptides (Keegstra et al., 1989, von Heijne et al., 1989). A plastid signal sequence (PTS) was an expected feature for a putative linalool synthase, since these enzymes are often localized to leucoplasts of oil glands (Turner et al., 1999). With 53 amino acids, the length of the PTS of this putative *LIS* was consistent with plastid targeting signals of other monoterpene synthases that usually contain 50–70 amino acids (Bohlmann et al., 1998a). In comparison to the signal peptide of the previously isolated *L. angustifolia* *LIS*, the PTS of this new putative *Lxi-LIS* was 28 aa longer and shared a number of similar amino acids with a different *LIS*-PTS from *L. latifolia*. The similarity between the putative *Lxi-LIS*, the *L.*

angustifolia LIS and the *L. latifolia* LIS sequence was not unexpected. *L. x intermedia* is the result of a cross between *L. angustifolia* and *L. latifolia*. Thus, the *L. x intermedia* *Lxi-LIS* gene may be the result of gene shuffling, featuring a novel LIS with the *L. latifolia* signal peptide and a *L. angustifolia* coding sequence.

Despite the close similarity to other linalool synthases, evidence of the functionality of the isolated LIS is so far inconclusive. Recombinant expression of the cDNA clone was highly successful; however, the recombinant protein was insoluble, which likely inhibited enzyme function in the subsequent *in vitro* assay. Protein insolubility is a common problem encountered in the bacterial expression of monoterpene synthases, likely caused by an extended plastid targeting signal peptide (Bohlmann et al., 1998a). For instance, Landmann et al (2007) noticed that short signaling peptides up to 35 bp did not diminish the solubility of the heterologous expressed *L. angustifolia* LIS (Landmann et al., 2007). However, the longer signal peptides (between 50-70 bp in length) of an *L. angustifolia* limonene synthase caused insoluble protein aggregates *in vitro* (Landmann et al., 2007). On the other hand, Williams et al. (1998) demonstrated that the partial removal of this preprotein yielded a functional, recombinant limonene synthase (Williams et al., 1998). The 65 amino acid extension of the putative *L. x intermedia* LIS homolog likely caused the insolubility of the recombinant protein and thus inhibited protein function. Deletion of the entire signal peptide however, should improve future attempts to express the recombinant *L. x intermedia* LIS protein and prove its functionality.

4.5. LIS transcription correlates with linalool abundance during flower ontogeny

A comparison between LIS (and *Lxi-LIS*) transcription and linalool accumulation during lavender flower ontogeny showed that the abundance of LIS and *Lxi-LIS* messages was closely correlated with linalool concentration in both Munstead and Grosso throughout flower development. In Grosso, *Lxi-LIS* gene transcription paralleled linalool concentration at all stages of development, similar to previous studies in *Clarkia breweri* (Pichersky et al., 1994, Dudareva et al., 1996), which demonstrated a correlation between the transcription, enzyme activity and emission of LIS and linalool. The high correlation coefficient of 0.81 suggests a direct relationship between LIS mRNA abundance and linalool production, supporting the

current hypotheses that the production of some monoterpenes is regulated at the level of transcription.

In Munstead flowers, *LIS* transcription decreased towards the end of flower development while linalool levels remained high, suggesting that *LIS* transcription preceded linalool production and that linalool was stored beyond the time of peak linalool synthesis. This pattern was similar to findings by Raguso et al. (1999), who showed that *LIS* mRNA in *C. breweri* accumulated during the final days of bud maturation and preceded the peak emission of linalool by two days (Raguso and Pichersky, 1999). However, a low correlation coefficient of 0.58 between transcript abundance and linalool concentration suggest that other regulatory mechanisms (e.g. posttranscriptional or posttranslational regulation) influence linalool abundance in Munstead. Further investigations are required to determine the exact nature of the regulatory mechanisms that control linalool abundance in Munstead and determine possible differences in the regulation of linalool synthesis in *L. angustifolia* and *L. x intermedia*.

4.6. Conclusion and future prospects

The current study aimed to determine the monoterpene profile of lavender grown in the Okanagan and define regulatory factors that control monoterpene abundance in lavender. Therefore, three hypotheses were tested, i.e. (i) monoterpene abundance and essential oil yield are specific to the lavender species; (ii) monoterpene abundance and essential oil yield are regulated developmentally, and (iii) monoterpene abundance is regulated at the level of gene expression.

The initial comparison of oil from various lavender cultivars showed that ‘English Lavender’ (*Lavandula angustifolia*) oils were high in linalool, but low in camphor content and were characterized by overall low oil yield. In contrast, cultivars of the hybrid species *Lavandula x intermedia*, produced large amounts of oil, marked by a high abundance of linalool as well as high camphor content. Thus, these results confirmed the empirical paradigm that monoterpene composition and yield of lavender oil are specific to lavender species the essential oil is derived from.

Monitoring monoterpene abundance and essential oil production throughout lavender ontogeny demonstrated that monoterpene abundances changed with the developmental stage of the lavender tissue, and markedly differed between flowers and leaves. Flowers produced the

bulk of the essential oil with highest levels of linalool at the stage when 30%-70% of the flower spike was in bloom. In contrast, leaves produced little oil which was characterized by high abundances of camphor and borneol.

The molecular basis for the differential distribution of monoterpenes throughout ontogeny is unclear. Based on presented data, there is a strong indication for a direct relationship between the abundance of *LIS* transcript and linalool abundance, suggesting that linalool production is regulated through gene transcription. However, other, unknown regulatory factors are likely to influence linalool abundance and further investigations are necessary to determine the exact nature of monoterpene regulation in lavender.

Taking these results together, this study can guide local lavender farming in adjusting lavender harvest to times when the most oil, with the desired monoterpene profile is produced. On more general terms, knowing the factors that control monoterpene abundance and regulate essential oil quality are valuable for the broad field of today's flower and scent industry. For decades the flower industry has put every effort into improving flower colour, longevity, shape, size and disease resistance, paying scant attention to the flower's most emblematic attribute: its scent. In recent years, the importance of flower scent has experienced a major comeback and scented versions of ornamental plants are more and more requested. Linalool is a major scent determinant in numerous ornamentals, and contributes to the aroma and flavour of many fruits. The mechanism by which linalool abundance is regulated in different tissues or throughout development is still unclear. However, results presented here suggested that linalool abundance is in part regulated through the transcription of *LIS*. Together with the isolation of a putative *LIS* gene from *L. x intermedia* cv. Grosso this may form the basis for the genetic modification of *L. x intermedia* to artificially increase or stabilize linalool abundance.

Lastly, throughout this study lavender has proven to be valuable as a new and unique model system for the investigation of volatile plant secondary metabolites. Monoterpene synthesis and regulation have been studied in numerous *Lamiaceae*, e.g. mint, sage and thyme. Blooming of these species is traditionally undesirable and repressed due to their use as herbs, thus limiting the current knowledge about monoterpenes and monoterpene synthases to their function and regulation in vegetative tissue. Lavender on the other hand can be used to provide insight into the regulation of volatiles specific to flowers. Monoterpene research in lavender

may thus aid to increase our understanding of scent regulation in general and lead to ways to enhance scent in flowers or to engineer plants that produce novel scents.

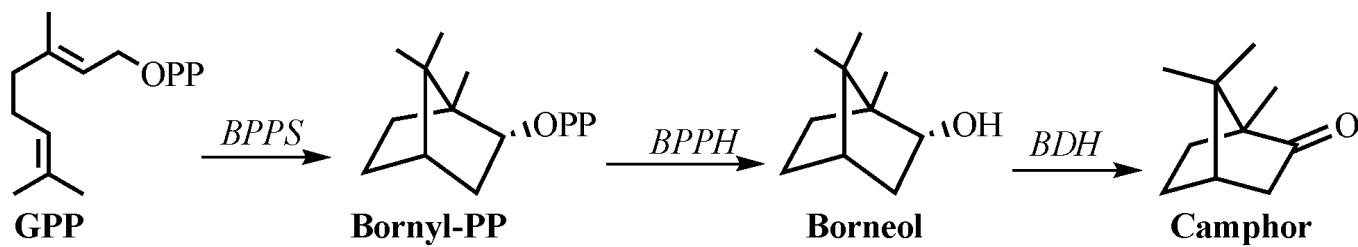


Figure 13: Putative camphor biosynthetic pathway. PP: -diphosphate; BPPS: Bornyl-diphosphate synthase, BPPH: Bornyl-diphosphate hydrolase, BDH: Borneol dehydrogenase

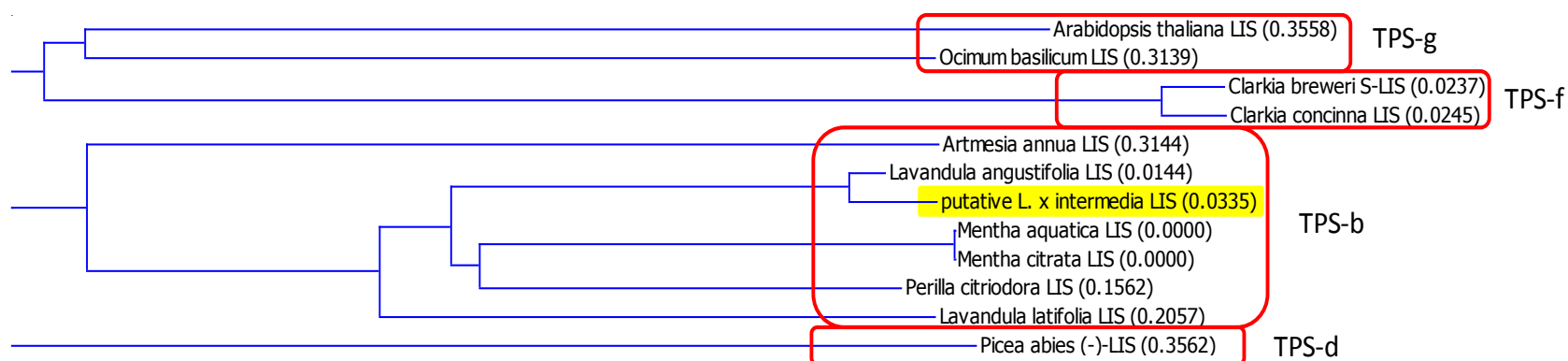


Figure 14: Neighbour-joining phylogenetic tree of selected linalool synthases. Amino acid sequences of published linalool synthases from various species were aligned to the putative LIS isolated from *L.x intermedia*. LIS belonging to identical terpene synthase classes (TPS-g, -f, -b, -d) are circled in red. *L.x intermedia* LIS, marked in yellow, belongs to the TPS-b class of terpene synthases (Bohlmann et al., 1997, Dudareva et al., 2003, Landmann et al., 2007).

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