

# Terpene Biosynthesis in Glandular Trichomes of Hop<sup>1,2[W][OA]</sup>

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Hop (*Humulus lupulus* L. Cannabaceae) is an economically important crop for the brewing industry, where it is used to impart flavor and aroma to beer, and has also drawn attention in recent years due to its potential pharmaceutical applications. Essential oils (mono- and sesquiterpenes), bitter acids (prenylated polyketides), and prenylflavonoids are the primary phytochemical components that account for these traits, and all accumulate at high concentrations in glandular trichomes of hop cones. To understand the molecular basis for terpene accumulation in hop trichomes, a trichome cDNA library was constructed and 9,816 cleaved expressed sequence tag (EST) sequences were obtained from random sequencing of 16,152 cDNA clones. The ESTs were assembled into 3,619 unigenes (1,101 contigs and 2,518 singletons). Putative functions were assigned to the unigenes based on their homology to annotated sequences in the GenBank database. Two mono- and two sesquiterpene synthases identified from the EST collection were expressed in *Escherichia coli*. Hop MONOTERPENE SYNTHASE2 formed the linear monoterpene myrcene from geranyl pyrophosphate, whereas hop SESQUITERPENE SYNTHASE1 (HISTS1) formed both caryophyllene and humulene from farnesyl pyrophosphate. Together, these enzymes account for the production of the major terpene constituents of the hop trichomes. HISTS2 formed the minor sesquiterpene constituent germacrene A, which was converted to  $\beta$ -elemene on chromatography at elevated temperature. We discuss potential functions for other genes expressed at high levels in developing hop trichomes.

Hop (*Humulus lupulus*) is a perennial, dioecious plant that belongs to the Cannabaceae family. "Hops" is the common term for the female inflorescences of hop plants, well known for their use in beer flavoring. These inflorescences develop into cones upon maturation. The lower parts of the inner surface of the bracts of mature female hop cones are covered with glandular trichomes, termed lupulin glands (Fig. 1). Glandular

trichomes, also referred to as secretory or peltate trichomes, are lipophilic glands comprising a group of secretory cells and a cuticle-enclosed cavity that fills with the secreted compounds (Oliveira and Pais, 1990; Saito et al., 1995). The plastids in glandular trichomes have less-defined membrane structures than chloroplasts and may be associated with synthesis and/or secretion of secondary metabolites, such as terpenoids and flavonoids (Oliveira and Pais, 1990).

Three major classes of secondary metabolites are synthesized and accumulated in hop lupulin glands; essential oils, bitter acids, and prenylflavonoids. Commercial hop varieties often differ in the content of these components, which determines their use in bittering and finishing (adding flavor and aroma) of beer. Essential oils are the principal aroma components of hops. Essential oils make up 0.5% to 3% (v/w) of the whole hop cone, and terpenoids are abundant in this fraction (Eri et al., 2000), accounting for up to 90% of the oil. The composition of essential oils is characteristic of the hop genotype and, together with that of bitter acids and flavonoids, has been used for distinguishing different hop varieties. In addition to hydrocarbon compounds, which are predominantly terpenes, oxygenated compounds and small amounts of sulfur-containing compounds are also found. The major monoterpene and sesquiterpene components of hop essential oils are myrcene,  $\alpha$ -humulene, and  $\beta$ -caryophyllene (Bernotiene et al., 2004). Most studies of hop terpenes have analyzed whole hop cones and

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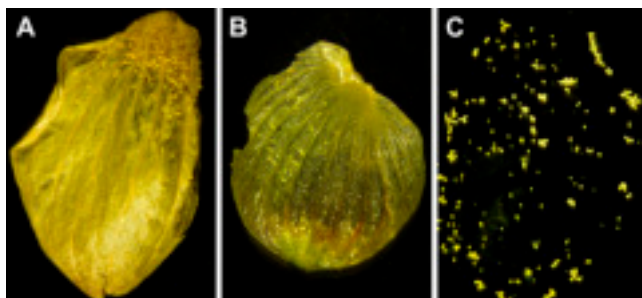
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**Figure 1.** Isolation of glandular trichomes from female hop bracts. A, Glandular trichomes at the base of the female bracteole. B, Female bracteole after trichome isolation. C, Isolated glandular trichomes.

there is little information on the content of these compounds in other tissues. It is also not clear whether the trichome is the exclusive organ for their biosynthesis and storage.

The bittering agents in beer are called bitter acids, which account for 10% to 20% of the hop cone by dry weight. The two representative bitter acids,  $\alpha$ -acid and  $\beta$ -acid (humulone and lupulone), are prenylated polyketide derivatives. Prenylated flavonoids have also been identified from hops and beer and include the two prenylchalcones xanthohumol and desmethylxanthohumol and the three prenylflavanones isoxanthohumol, 8-prenylnaringenin, and 6-prenylnaringenin. Prenylchalcones and bitter acids accumulate at low levels at the onset of flowering and their concentrations gradually increase during the development of female hop cones (De Keukelere et al., 2003). The *in vitro* prenylation of the aromatic intermediates in the biosynthesis of bitter acids has been described using a crude hop extract (Zuurbier et al., 1998). In contrast to membrane-bound (iso)flavonoid prenyltransferases (Welle and Grisebach, 1991; LaFlamme et al., 1993; Sasaki et al., 2008), the hop bitter acid prenyltransferase activities were in the soluble fraction of the protein extract (Zuurbier et al., 1998). However, the bitter acid prenyltransferase remains to be characterized and it is not clear whether prenylflavonoids and bitter acids are synthesized by the same soluble prenyltransferase or whether polyketide prenylation is functionally related to other areas of terpene metabolism in hop trichomes.

In one of the first examples of applying genomics approaches to plant secondary metabolism, sequencing of a peppermint (*Mentha piperita*) oil gland cDNA library proved highly effective for studying essential oil biosynthesis in peppermint glandular trichomes (Lange et al., 2000). A similar approach has been reported recently with hop trichomes, leading to identification of an *O*-methyltransferase active in the biosynthesis of prenyl chalcone (Nagel et al., 2008). Essential oils, bitter acids, and prenylflavonoids are all derived from pathways of terpene metabolism, but the enzymes responsible for these processes have yet to be identified in hops. To facilitate gene discovery in hop natural product biosynthesis, a cDNA library was

constructed from total RNA isolated from lupulin glands, and candidates for diverse terpene biosynthetic enzymes identified based on sequence similarities to previously known genes and direct functional identification through expression of recombinant terpene synthases in *Escherichia coli*. We here identify the mono- and sesquiterpene synthases involved in the formation of myrcene, humulene, and caryophyllene, and also describe the most highly expressed genes in the metabolically specialized hop trichomes.

## RESULTS

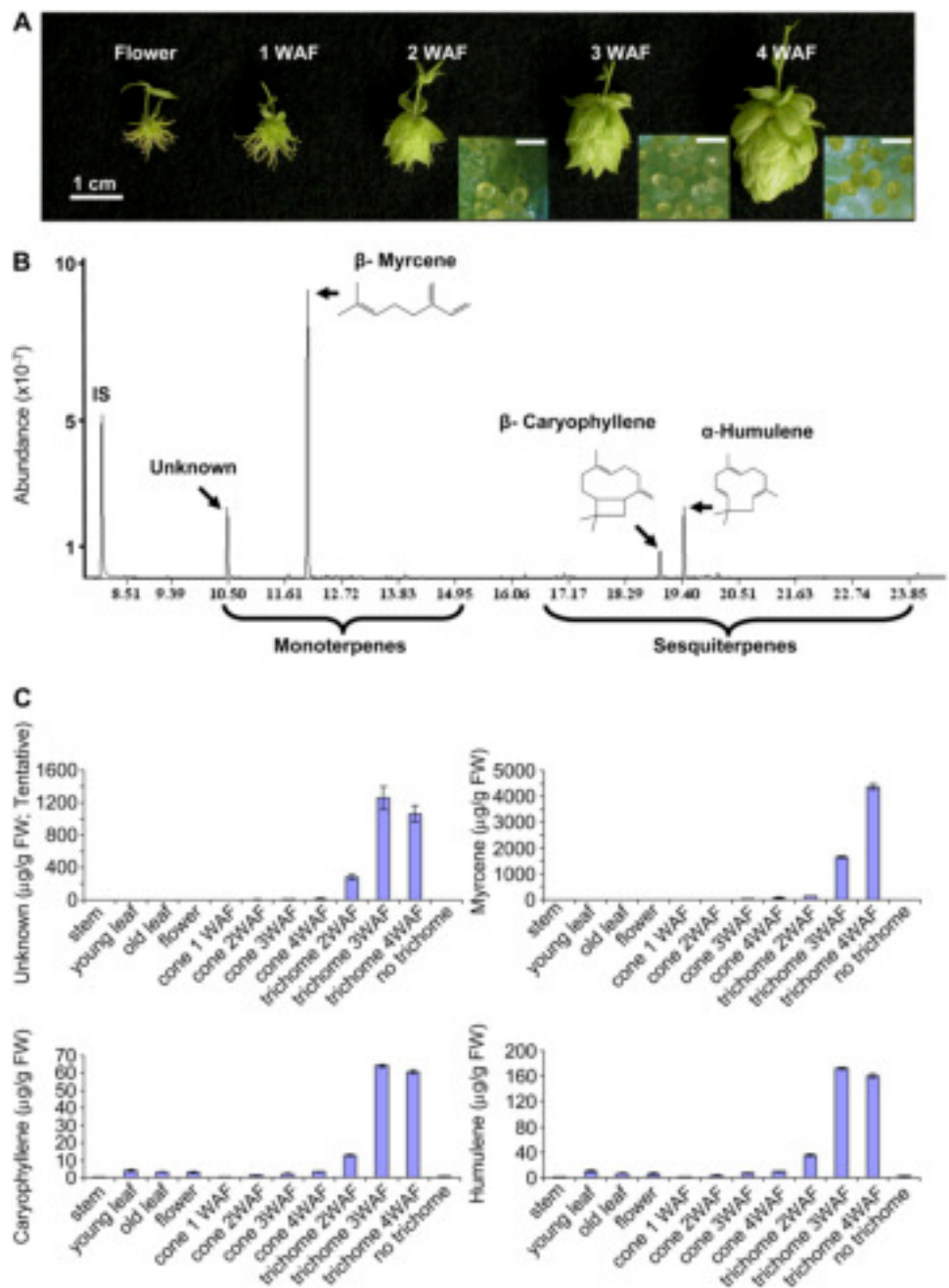
### Terpene Content and Composition of Hop Glandular Trichomes

Hexane extracts of different tissues from hop cultivar Nugget were collected and analyzed by gas chromatography (GC)-mass spectrometry (MS). This revealed that the linear monoterpene myrcene and an unidentified compound of retention time 10.491 min were found exclusively in trichomes. MS analysis was most consistent with a structure of 2,7-dimethyl-1,6-octadiene ( $C_{10}H_{18}$ ) for the unidentified compound, which accounted for approximately 15% ( $n = 3$  biological replicates) of the total monoterpenes in the trichomes. The levels of both compounds increased during trichome development. In the trichomes of 4-week-old hop cones, myrcene comprised about 80% ( $n = 3$ ) of the monoterpenes based on the area of peaks with monoterpene-specific fragments (10–15 min,  $m/z$  136, 121, 93, 69) resolved by GC (Fig. 2). Other monoterpenes, such as linalool (retention time 14.007 min), were present in trace amounts and accumulated mainly in floral tissue (data not shown).

Humulene and caryophyllene were the two major sesquiterpenes in isolated hop trichomes. Together, they account for approximately 85% ( $n = 3$ ) of the total sesquiterpenes in the trichomes (16.5–24 min, peaks with sesquiterpene-specific fragments of  $m/z$  204, 161, 93, 69; Fig. 2). However, unlike myrcene and 2,7-dimethyl-1,6-octadiene (tentative), humulene and caryophyllene were not specific to trichomes and were also found in other tissues, such as leaves and flowers. Moreover, the ratios of humulene to caryophyllene in different tissues were almost identical, at about 3.0 based on peak areas (Fig. 2). This suggests the possibility that they may be formed by the same terpene cyclase enzyme (see below).

We also extracted the terpenoids from hop trichomes with ethyl acetate in place of hexane. This resulted in overall extraction of more terpenoids as determined by GC-MS and comparison to the internal standard (10% more myrcene and about 35% more humulene and caryophyllene), but the profiles included some nonterpenoid compounds eluting at higher retention times. Importantly, the patterns of terpenoid compounds were very similar using the two different extraction protocols and the ratio of humulene to caryophyllene was the same.

**Figure 2.** Accumulation of mono- and sesquiterpenes in trichomes and other tissues of hop. A, Developmental sequence of developing hop flowers and trichomes. Bars on trichome photographs are 200  $\mu\text{m}$ . WAF, Weeks after flowering. B, GC-MS analysis of terpenoids from mature trichomes from female bracts of hop cultivar Nugget. IS, Internal standard (toluene). C, Levels of unidentified compound (retention time 10.491 min; see B), myrcene, caryophyllene, and humulene in different tissue and in trichomes at different stages of development.



### Construction, Annotation, and Functional Classification of a Hop Glandular Trichome EST Library Unigene Set

The Phoenix variety used for generation of the cDNA library has high essential oil and  $\alpha$ -acid content and was developed in the UK as a dual-purpose hop for both bittering and finishing. It has 8% to 12%  $\alpha$ -acids, 4.2% to 5.5%  $\beta$ -acids, 0.54% xanthohumol, and a total essential oil percentage of 1.2% to 2.5%.

Glandular trichomes were collected from female bracts (Fig. 1). The conventional method for cDNA library construction requires impractically large

amounts of trichomes to extract sufficient quantities of mRNA. We therefore employed a PCR-based cDNA library construction method that uses small amounts of total RNA as starting material; 12,665 single-pass EST sequences were generated by random sequencing of 16,152 cDNA clones. Vector, low-quality, and short sequences (less than 100 bp) were excluded by using the cross-match program (<http://www.phrap.org/phredphrapconsed.html>) along with manual curation. A high percentage of ribosomal RNA contamination was observed and the corresponding sequences were

removed during postsequencing cleansing of the ESTs, resulting in 9,816 cleansed high-quality EST sequences.

An EST database was generated containing the 9,816 sequences assembled into 1,101 contigs and 2,518 singletons. The contigs and singletons are collectively referred to as unigenes. The unigenes were searched against the National Center for Biotechnology Information (NCBI) nonredundant protein database (released on 9/30/07) using the BLASTX algorithm, and annotated according to their homologous sequences in the GenBank database. The most abundant unigenes are shown in Table I, and unigenes encoding enzymes of terpene and prenylflavonoid biosynthesis are listed in Table II and their frequencies depicted diagrammatically on metabolic pathways in Figure 3.

In addition to annotation based on sequence similarities to NCBI database entries, the unigenes were also classified into three major functional categories—cellular component, molecular function, and biological process—according to the standard Gene Ontology (GO; [www.geneontology.org](http://www.geneontology.org)) terms, by searching the GO standard protein database released in August 2007 (BLASTX,  $e$ -value  $< 1 \times 10^{-4}$ ; Supplemental Fig. S1). Of the cellular component GO terms, 44% and 34% of hop unigenes were related to cell part and organelle, respectively. In the category of molecular function, 47% of hop unigenes were involved in catalytic activity and 26% in binding activity. Under the category of biological process, 31% and 30% were involved in cellular process and metabolic process, respectively, indicating that the hop glandular trichomes are highly metabolically active. The hop glandular trichome unigene sequences and GO classifications were deposited in the TrichOME database along with trichome EST sequences from other species and are publicly available for viewing and searching (<http://trichome.noble.org/trichomedb>).

### The Most Abundant Hop Trichome Unigenes

The 15 most highly represented unigenes in the hop glandular trichome cDNA library are listed in Table I. The abundance of their transcripts suggests critical roles in secondary metabolite synthesis and transport and in glandular trichome development. Indeed, four of these highly represented unigenes encoded enzymes involved in secondary metabolite synthesis, namely, valerophenone synthase (VPS), chalcone synthase (CHS), chalcone isomerase (CHI)-like protein, and isopentenyl diphosphate (IPP) isomerase. Unigene TCHL10255 encoded an AMP-dependent synthetase and ligase family protein (also known as acyl-activating enzyme; Shockey et al., 2003). Acyl-activating enzymes convert carboxylic acids to acyl-AMP intermediates and then to acyl-CoAs, and could potentially be involved in the formation of branched-chain acyl-CoAs as substrates for VPS.

ESTs homologous to food allergens and nonspecific lipid transfer proteins (LTPs) were also highly expressed in hop glandular trichomes. Trichomes are also known for their roles in storage and secretion of heavy metals and in defense (Küpper et al., 2000; Choi et al., 2001), and unigene TCHL10880 (containing 120 ESTs) was similar to metallothioneins and TCHL10947 (composed of 137 ESTs) was homologous to cystatin. Metallothioneins are heavy-metal binding proteins that play dual roles in heavy-metal detoxification and metal ion uptake/transport, and cystatin is a Cys protease inhibitor that is induced by biotic and abiotic stresses. TCHL10134 showed sequence similarity to a tobacco (*Nicotiana tabacum*) senescence-related protein, the expression of which was transiently increased upon bacterial (*Rhodococcus fascians*) infection (Simon-Mateo et al., 2006), suggesting a potential role in pathogen defense.

**Table I.** The top 15 most abundant unigenes in the hop glandular trichome cDNA library

Unigene	No. ESTs	BLAST Hit	GenBank ID	$E$ Value
TCHL10783	194	Cytochrome P450-like protein ( <i>Nicotiana tabacum</i> )	BAA10929	4e-86
TCHL10811	184	VPS ( <i>Humulus lupulus</i> )	O80400	0
TCHL10806	167	Major allergen Pru p1 ( <i>Prunus persica</i> )	ABB78006	9e-56
TCHL10947	137	Cystatin-like protein ( <i>Citrus × paradisi</i> )	AAG38521	6e-28
TCHL11024	123	Putative CHI ( <i>Lycopersicon esculentum</i> )	AAQ55182	7e-64
TCHL10880	120	Metallothionein 1a ( <i>Populus balsamifera</i> subsp. <i>trichocarpa</i> × <i>Populus deltoides</i> )	AAT02522	0.008
TCHL10775	114	Gly-rich protein ( <i>Citrus unshiu</i> )	BAA92155	1e-06
TCHL10835	114	Short-chain dehydrogenase/reductase (SDR) family protein ( <i>Arabidopsis thaliana</i> )	NP_567300	2e-59
TCHL10005	85	Selenium-binding protein ( <i>Medicago sativa</i> )	CAC67501	5e-43
TCHL10130	58	CHS (CHS2) ( <i>Humulus lupulus</i> )	BAB47194	0
TCHL10382	56	Nonspecific lipid transfer-like protein ( <i>Prosopis juliflora</i> )	ABF06565	1e-32
TCHL10509	51	Isopentenyl pyrophosphate isomerase ( <i>Pueraria montana</i> var. <i>lobata</i> )	AAQ84167	2e-122
TCHL10255	32	AMP-dependent synthetase and ligase family protein ( <i>Arabidopsis thaliana</i> )	NP_179356	1e-75
TCHL10134	32	Senescence-associated protein ( <i>Nicotiana tabacum</i> )	AAZ23261	2e-44
TCHL10384	32	Type 2 peroxiredoxin ( <i>Brassica rapa</i> subsp. <i>pekinensis</i> )	AAD33602	4e-63

**Table II.** *Hop unigenes encoding enzymes of terpene and prenylflavonoid/bitter acid biosynthesis*

Unigene	No. ESTs	BLAST Hit	GenBank ID	E Value
TCHL10811	184	VPS ( <i>Humulus lupulus</i> )	O80400	0
TCHL10130	58	CHS (CHS2) ( <i>Humulus lupulus</i> )	BAB47194	0
TCHL10129	23	CHS-like protein (CHS4) ( <i>Humulus lupulus</i> )	CAD23044	0
TCHL10662	10	CHS (chs_H1) ( <i>Humulus lupulus</i> )	CAK19318	1e-149
TCHL10849	5	Putative orcinol O-methyltransferase ( <i>Rosa odorata</i> )	CAJ65661	1e-80
TCHL10548	6	1-Deoxy-D-xylulose-5-P synthase ( <i>Pueraria montana</i> )	AAQ84169	4e-53
TCHL10613	2	1-Deoxy-D-xylulose-5-P reductoisomerase ( <i>Mentha</i> × <i>piperita</i> )	AAD24768	1e-28
TCHL10661	9	2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase	–	–
TCHL10150/ 11107	14	Hydroxymethylbutenyl-4-diphosphate synthase	–	–
TCHL10138/ 10273	22	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase ( <i>Arabidopsis thaliana</i> )	NP_567965	1e-47
	19	–	–	–
TCHL10436	4	PAL1 ( <i>Prunus avium</i> )	AAC78457	2e-155
TCHL10998	2	PAL2 ( <i>Rubus idaeus</i> )	AAF40224	3e-51
ES654594	1	PAL ( <i>Populus kitakamiensis</i> )	BAA21643	3e-35
EX516754	1	PAL3 ( <i>Manihot esculenta</i> )	AAK60273	1e-40
TCHL10666	9	C4H ( <i>Citrus</i> × <i>paradisi</i> )	AAK57011	3e-96
TCHL10606	2	C4H (CYP73) ( <i>Catharanthus roseus</i> )	CAA83552	1e-96
ES652343	1	C4H ( <i>Gossypium arboreum</i> )	AAG10197	5e-63
EX519255	1	4CL ( <i>Arabidopsis thaliana</i> )	AAP03021	1e-27
EX520059	1	4CL-like protein ( <i>Arabidopsis thaliana</i> )	AAP03022	4e-97
TCHL10609	2	Pinene synthase ( <i>Quercus ilex</i> )	CAK55186	2e-53
TCHL10730	2	(+)- $\delta$ -Cadinene synthase ( <i>Gossypium arboreum</i> )	CAA77191	9e-14
TCHL10281	6	Sesquiterpene cyclase ( <i>Artemisia annua</i> )	AAG24640	4e-03

### Biosynthesis of Early Terpene Pathway Precursors

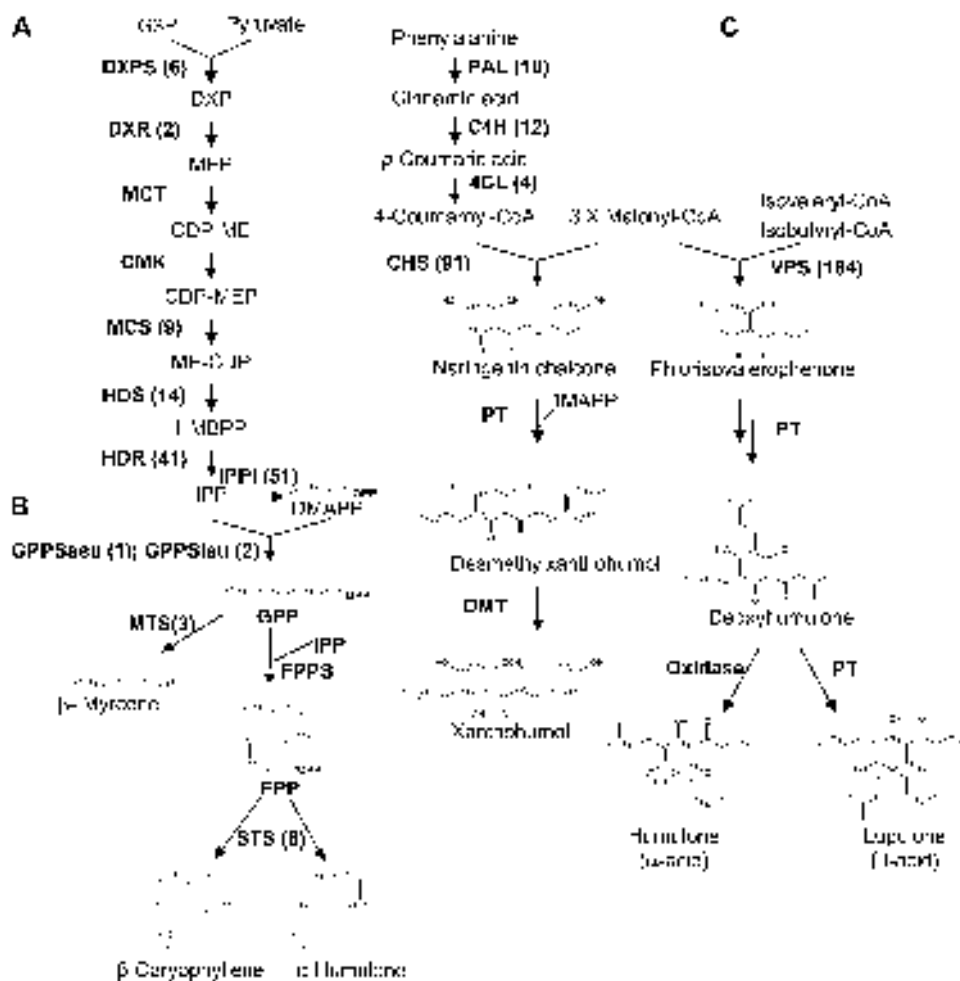
Hop bitter acids and prenylflavonoids are formed by the transfer of one or more single prenyl (dimethylallyl diphosphate; DMAPP) groups to a polyketide acceptor molecule, and mono- and sesquiterpenes are derived from longer chain allylic pyrophosphates formed from DMAPP/IPP. Both the cytosolic mevalonate (MVA) pathway and the plastidial 2-C-methyl-D-erythritol-4-P (MEP) pathway can produce IPP, which is then converted to its allylic isomer, DMAPP, through the action of IPP isomerase (Lichtenthaler, 1999). Based on the stable isotope-labeling pattern of dimethylallyl groups in humulone, it was concluded that hop bitter acids are derived from the MEP pathway (Goese et al., 1999). Consistent with these labeling results, unigenes encoding the MEP pathway enzymes 1-deoxy-D-xylulose-5-P synthase, 1-deoxy-D-xylulose-5-P reductoisomerase, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, and 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase, were present in the hop glandular trichome cDNA library (Fig. 3; Table II). ESTs encoding 2-C-methyl-D-erythritol-4-P cytidyltransferase and 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase were not identified, indicating that, although abundant in biosynthetic genes, our hop unigene set did not contain all of the transcripts for secondary metabolite synthesis in glandular trichomes. In a recent report on a hop trichome EST collection from cultivars Taurus and Nugget, both of these enzymes were identified,

although the libraries in this case were a mixture of normalized and non-normalized (Nagel et al., 2008). It should, however, be noted that no ESTs corresponding to any of the enzymes of the MVA pathway were represented in the present collection.

Hop essential oils are synthesized from geranyl diphosphate (GPP) and farnesyl diphosphate (FPP). A cDNA encoding FPP synthase (FPPS) was cloned and characterized from hop that showed both GPP synthase and FPPS activities, but not prenyltransferase activity toward phlorisovalerophenone (PIVP) in bitter acid synthesis (Okada et al., 2001). We could not identify this FPPS gene or its homolog in our glandular trichome cDNA library. Unigenes homologous to GPP synthase large and small subunits were, however, present in the EST database (data not shown), and the biochemistry of the heterodimeric hop GPP synthase will be described elsewhere.

### Functional Identification of Hop Monoterpene Synthases

A search of the hop trichome EST database for potential terpene synthases revealed three different unigenes with high protein sequence identity to functionally identified monoterpene synthases (designated as HlMSTS1, HlMSTS2, and HlMSTS3). The EST clones of HlMSTS1 and HlMSTS2 were found to be truncated at their N termini. 5'-RACE was therefore used to clone the corresponding full-length cDNAs for these two putative monoterpene synthases. The full-length



**Figure 3.** Biosynthetic pathways for terpene-derived natural products found in hop trichomes, showing EST abundance in the hop glandular trichome cDNA library. **A**, The MEP pathway leading to DMAPP biosynthesis. **B**, General reactions of mono- and sesquiterpene biosynthesis. **C**, Prenylflavonoid and bitter acid biosynthesis. Chemical structures of selected compounds are shown. The abundance of ESTs corresponding to each biosynthetic gene in the hop glandular trichome cDNA library is indicated in parentheses. DXPS, 1-Deoxy-D-xylulose-5-P synthase; DXR, 1-deoxy-D-xylulose-5-P reductoisomerase; MCT, 2-C-methyl-D-erythritol-4-P cytidyltransferase; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MCS, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; HDS, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase; HDR, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate reductase; IPP, isopentenyl diphosphate/dimethylallyl diphosphate isomerase; OMT, *O*-methyltransferase; PT, prenyltransferase. Numbers in parentheses represent the number of occurrences of an EST corresponding to the particular enzyme in the 9,816 cleansed hop ESTs.

cDNA of HIMTS1 (2,095 bp) encodes a peptide sequence of 585 amino acids with a calculated molecular mass of 67,510 D and a pI of 4.9. The full-length cDNA of HIMTS2 (1,953 bp) contains an open reading frame of 1,842 nucleotides that encode a predicted protein of 613 amino acids with a pI of 5.68. HIMTS1 and HIMTS2 share 46.7% amino acid identity to each other. Both have a plastid-targeting peptide at the N terminus (the first 31 and 46 amino acids for MTS1 and MTS2, respectively; predicted with TargetP 1.1 software). The deduced proteins of HIMTS1 and HIMTS2 show 48.9 and 52% amino acid identity with *Vitis vinifera* ( $\alpha$ )-terpineol synthase (Martin and Bohlmann, 2004), respectively (Fig. 4A). According to BLAST results, HIMTS3 is an ortholog of linalool synthase, and was highly expressed in flower tissues where linalool accumulation was highest. We did not attempt to further characterize HIMTS3.

Truncated forms of HIMTS1 and HIMTS2 with the predicted plastid-targeting peptides removed were expressed in *E. coli*. After induction of protein expression with isopropylthio- $\beta$ -D-galactoside at 16°C for 16 h, crude bacterial extracts were used for terpene synthase assays using GPP (for monoterpene syn-

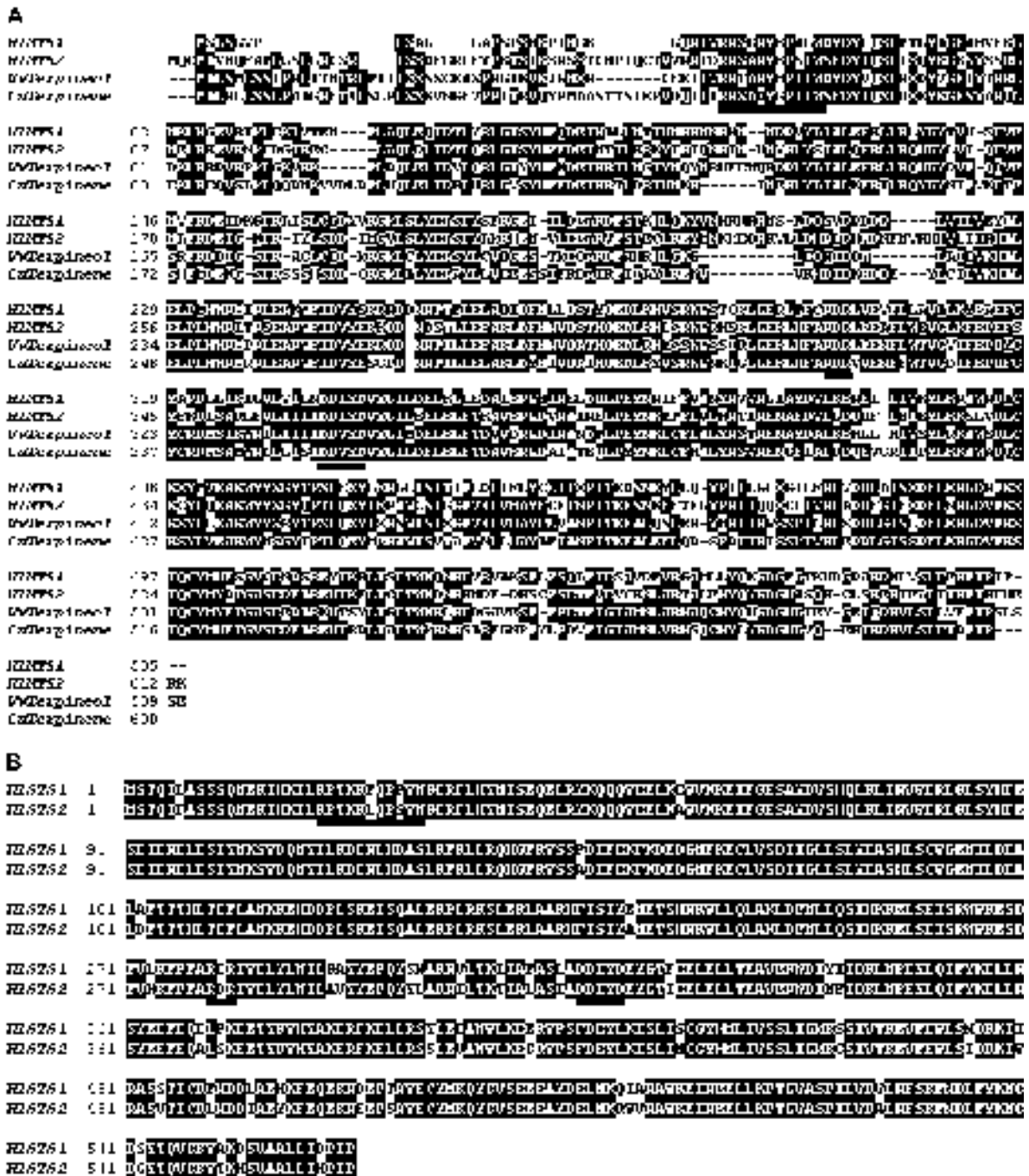
thase), FPP (for sesquiterpene synthase), and geranylgeranyl diphosphate (GGPP; for diterpene synthase) as substrates. GC-MS analysis showed that myrcene was the only product when the extract containing HIMTS2 was incubated with GPP (Fig. 5A). No product could be detected with FPP or GGPP as substrates. These results therefore indicate that HIMTS2 activity leads to the formation of myrcene in hop trichomes. We were unable to detect monoterpene, sesquiterpene, or diterpene synthase activity in extracts containing HIMTS1.

Kinetic analysis of purified His-tagged recombinant HIMTS2 revealed a  $K_m$  value for geranyl pyrophosphate of  $7.65 \pm 2.40 \mu\text{M}$  ( $n = 3$ ).

#### Functional Identification of Hop Sesquiterpene Synthases

ESTs corresponding to two different sesquiterpene synthase-like genes (designated as HISTS1 and HISTS2) were also identified from the hop trichome library. The full-length cDNAs of HISTS1 and HISTS2 were obtained using 5'-RACE. The full-length cDNA of HISTS1 (1,842 bp) encodes a peptide se-



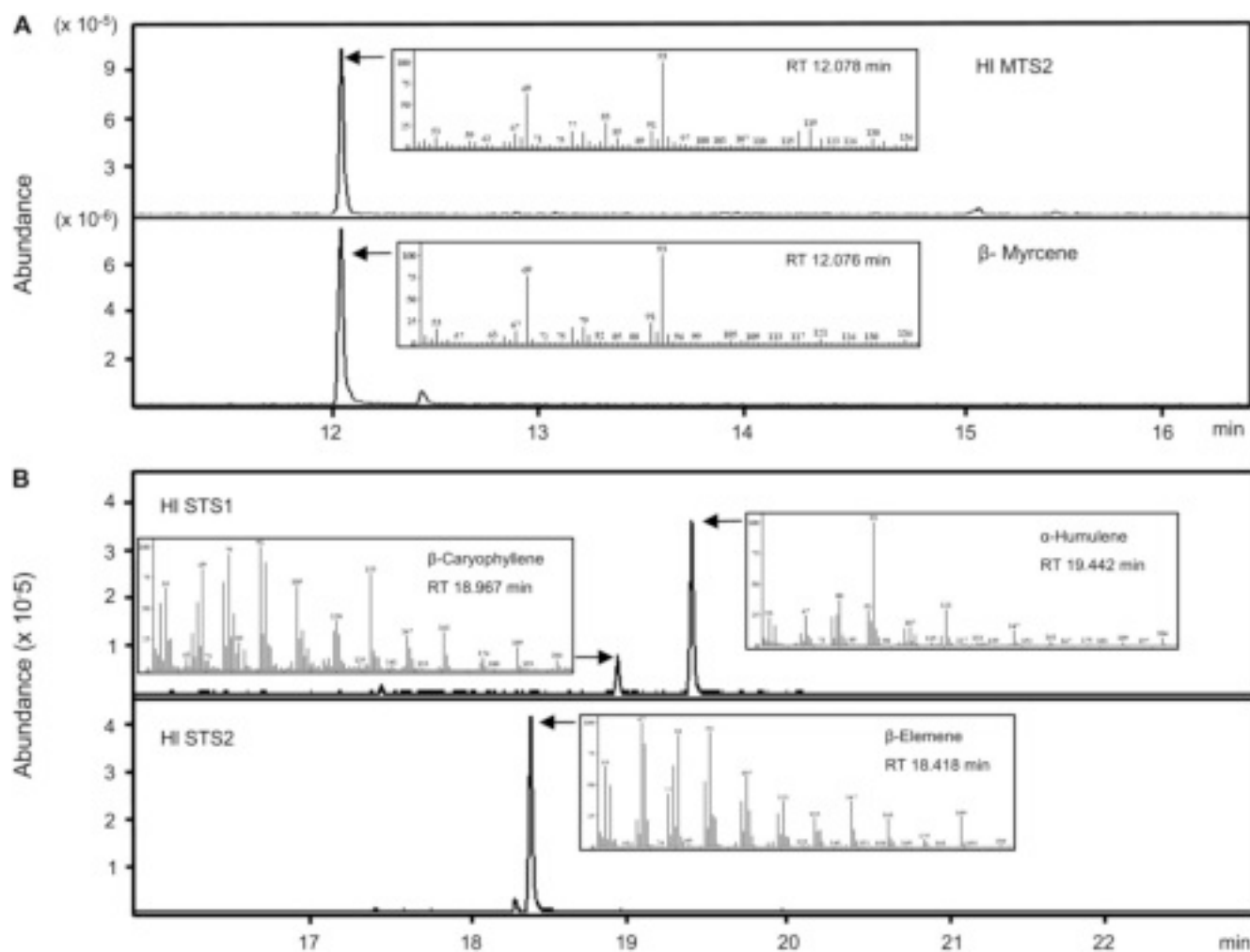


**Figure 4.** Alignments of hop MTS (A) and STS (B) sequences. Other MTS sequences are from *Citrus unshiu* (accession no. BAD27259) and *V. vinifera* (AAS79352). Identical amino acids are shown as white letters on a black background. The RR(P) $X_8$ W, RXR, and DDXXD motifs are underlined.

quence of 563 amino acids with a calculated molecular mass of 66,218 D and a pI of 5.1. The full-length cDNA of HISTS2 (1,863 bp) contains an open reading frame of 1,692 nucleotides that encode a predicted protein of 563 amino acids with a pI of 5.44. HISTS1 and HISTS2 share 92.4% identity to each other at the protein level (Fig. 4B) and 50% amino acid identity with a function-

ally characterized *V. vinifera* (-)-germacrene D synthase (Lücker et al., 2004).

HIMTS1, HIMTS2, HISTS1, and HISTS2 all contain the RR(P) $X_8$ W, RXR, and DDXXD (X is any amino acid) motifs, which are key features of most angiosperm terpene synthases (Keeling and Bohlmann, 2006; Fig. 4).



**Figure 5.** GC-MS analysis of enzymatic products from recombinant hop mono- and sesquiterpene synthases. A, Portion of chromatogram showing the product of the reaction of GPP with HIMTS1 and its corresponding mass spectrum (inset). Bottom, Same for an authentic standard of myrcene. B, Portion of chromatogram showing the two products of the reaction of FPP with HISTS1 and their corresponding mass spectra (inset). Bottom, Same for HISTS2.

HISTS1 and HISTS2 were expressed in *E. coli* using the same strategy as employed for the monoterpene synthases. Both enzymes were active with FPP as substrate, but not with GPP or GGPP. Terpenoid products were identified by GC-MS. The major sesquiterpene products of HISTS1 were identified as humulene (70% of total products) and caryophyllene (25%; Fig. 5B). This ratio of humulene to caryophyllene was similar to the value in the essential oil of the hop trichomes.

In spite of the very close sequence identity between HISTS1 and HISTS2, the products of HISTS2 are not humulene and caryophyllene (Fig. 5B). The major product of HISTS2 was shown by GC-MS analysis to be  $\beta$ -elemene, a relatively minor component of hop essential oil. However,  $\beta$ -elemene can be formed *in vitro* by the rearrangement of germacrene A at high temperatures, such as used in the present GC-MS analysis. We therefore analyzed the product of the

STS2 enzymatic reaction by GC-MS using lower injection temperatures (150°C and 180°C instead of the usual 280°C).  $\beta$ -Elemene was no longer observed and was replaced by a broad peak corresponding to germacrene A).

Kinetic analysis of purified His-tagged recombinant HISTS1 and HISTS2 revealed  $K_m$  values for farnesyl pyrophosphate of  $0.70 \pm 0.07$  ( $n = 3$ ) and  $0.49 \pm 0.04$  ( $n = 3$ )  $\mu\text{M}$ , respectively.

#### Tissue-Specific and Developmental Expression of Hop Terpene Synthases

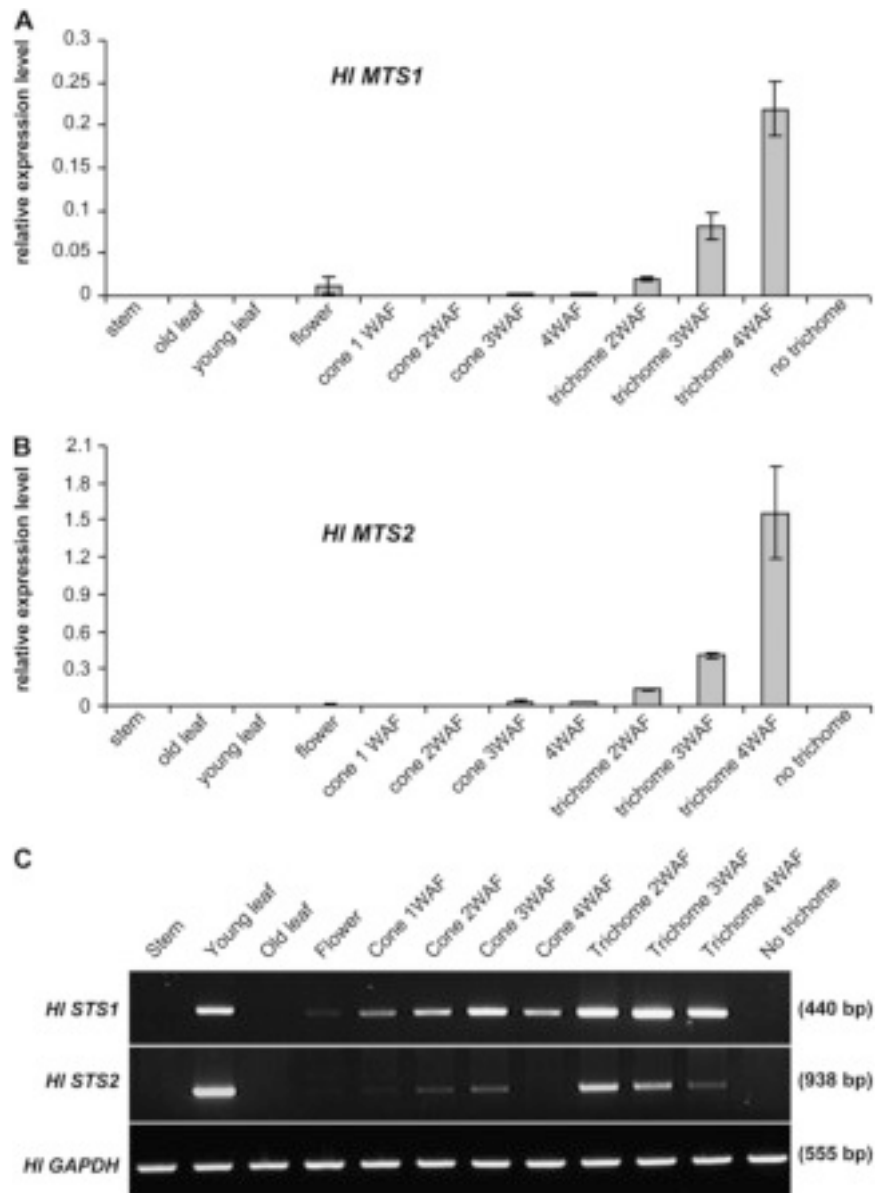
To test whether the patterns of terpene metabolite production in hops can be explained by the expression of the above-characterized terpene synthases, real-time PCR was first performed to examine the expression of HIMTS1 and HIMTS2 in different tissues and in trichomes at different developmental stages. HIMTS1



and HIMTS2 transcript levels followed the same developmental pattern as the monoterpene metabolites (Fig. 6, A and B). Both HIMTS1 and HIMTS2 transcript levels were highest in trichomes from cones at 4 weeks after flowering (Fig. 6, A and B).

Because of the high sequence identity between HISTS1 and HISTS2, we could not find good primers for real-time reverse transcription (RT)-PCR to distinguish between the two genes. Semiquantitative RT-PCR was therefore used to analyze the expression patterns of HISTS1 and HISTS2. HISTS1 transcripts were abundant in those tissues with high levels of humulene and caryophyllene, and also paralleled the levels of these compounds in hop trichomes during development. HISTS2 transcripts were abundant in young leaf tissue, although they were also detected in other tissues (Fig. 6C).

**Figure 6.** Tissue-specific expression of hop terpene synthases. A, Quantitative real-time PCR analysis of HIMTS1 transcript levels in different hop tissues and different developmental stages of cones and trichomes. No trichome = mature cones with trichomes removed. B, As above, for HIMTS2 transcripts. C, Semiquantitative RT-PCR analysis of HISTS1 and HISTS2 transcripts in different hop tissues and different developmental stages of cones and trichomes.



### Polyketide Biosynthesis and Prenylation

Hop prenylflavonoids are formed by transfer of a 5-carbon prenyl group to a chalcone precursor, itself formed by the condensation of malonyl-CoA and 4-coumaroyl-CoA by CHS. 4-Coumaroyl-CoA is derived from L-Phe by the sequential actions of L-Phe ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL). Four PAL gene homologs were present in the hop glandular trichome cDNA library (Table II), and three unigenes were similar to C4H from different plant species; TCHL10606 matched to the N terminus of the enzyme, whereas the closely related TCHL10666 and the singleton ES652343 matched to the C terminus. Arabidopsis (*Arabidopsis thaliana*) 4CL-like protein homologs were also found in the cDNA library.

Bitter acid and prenylflavonoid biosynthesis share several features, particularly the involvement of a polyketide synthase and subsequent prenyltransferases. The polyketide synthase for bitter acid biosynthesis, *VPS*, is related to *CHS*, and produces PIVP from malonyl-CoA and isovaleryl-CoA. *VPS* and *CHS* from hop have been cloned and functionally characterized (Paniego et al., 1999; Okada and Ito, 2001; Okada et al., 2004). In contrast to a single *VPS* gene in hops, *CHS* constitutes a family of four genes, *CHS2*, *CHS3*, *CHS4*, and *CHS\_H1*. *VPS* and *CHS2* were among the most abundant unigenes in the present hop glandular trichome cDNA library, represented by 184 and 58 ESTs, respectively (Table I). *CHS4* and *CHS\_H1* were also present, whereas *CHS3* was not identified (Table II).

Plant CHIs are classified into four subfamilies according to their phylogenetic relationship (Ralston et al., 2005). Proteins in the CHI3 and CHI4 subfamilies show sequence similarity to previously characterized CHIs, but have not been biochemically characterized to date and are therefore described as CHI-like proteins. HICHI-like 1, which was among the most abundant unigenes in the glandular trichome cDNA library (Table I), was homologous to a tomato (*Solanum lycopersicum*) CHI-like protein (LeCHI-like) that belongs to the CHI3 subfamily, and HICHI-like 2 was similar to an Arabidopsis CHI-like protein (At5g05270) that belongs to the CHI4 subfamily. Neither HICHI-like 1 nor HICHI-like 2 exhibited CHI activity when expressed as recombinant proteins in *E. coli* (data not shown).

Transfer of DMAPP to polyketide acceptor molecules is likely catalyzed by aromatic prenyltransferases in hops (Stevens and Page, 2004). Aromatic prenyltransferases have been isolated and characterized from bacteria and plants and can be grouped into two classes: the soluble bacterial prenyltransferases and the membrane-bound bacterial and plant UbiA family prenyltransferases (Sasaki et al., 2008; Tian et al., 2008). The UbiA family prenyltransferases share the common motif (N/D)DXXD for prenyl diphosphate binding (Bräuer et al., 2004). By searching hop unigenes with the prenyl diphosphate-binding motif, a single unigene was identified that showed strong sequence similarity to a membrane-bound prenyltransferase involved in ubiquinone synthesis (data not shown).

## DISCUSSION

The generation and sequencing of an EST library from hop lupulin glands has enabled us to identify and characterize the enzymes involved in the biosynthesis of the major mono- and sesquiterpene aroma compounds produced in these trichomes. As in a previous study (Nagel et al., 2008), ESTs corresponding to MEP pathway enzymes were highly abundant as compared with MVA pathway transcripts. Although this suggests that the DMAPP/IPPP for all classes of terpene synthesis in the hop trichomes originates primarily from the MEP

pathway, it is perhaps dangerous to equate EST counts to expressed enzymatic activities. Nevertheless, the MEP pathway has previously been shown to provide precursors for both mono- and sesquiterpene biosynthesis in snapdragon (*Antirrhinum majus*) flowers (Dudareva et al., 2005).

On comparing the overall transcript abundances in our EST library with those in the two non-normalized libraries previously generated from trichomes of hop cultivars Taurus and Nuggett (Nagel et al., 2008), 47.5% of the ESTs (1,087 of 2,290) in the Taurus/Nuggett libraries could hit targets in the cultivar Phoenix library described in this article, and 33.4% of the Phoenix ESTs could hit targets in the Taurus/Nuggett libraries. The lack of closer coincidence could arise from differences in both hop cultivar and developmental stage. However, all of the terpene synthases identified in this article could be found in the Taurus/Nuggett libraries, and two of the three *O*-methyltransferases previously identified (Nagel et al., 2008) were also represented in the Phoenix library.

Myrcene, caryophyllene, and humulene represent the bulk of the terpene component of hop essential oil. The relative proportions of caryophyllene and humulene were very similar in all tissues in which these compounds were produced and the same as produced by recombinant HISTS1 in vitro. Furthermore, we did not observe one of the compounds without the other in any of the hop tissues analyzed.

HISTS1 and HISTS2 are 92.4% identical at the amino acid level, but make different products.  $\beta$ -Elemene, the initially measured product of HISTS2, has been previously described as a minor component of hop essential oils (Katsiotis et al., 1989), although, as shown here, it may be derived nonenzymatically from germacrene A. It is likely that the active-site cavities of HISTS1 and HISTS2 differ in size, shape, or polarity as a result of the few amino acids that are different between the two enzymes, leading to different modes of cyclization.

The previously described myrcene synthase from grand fir (*Abies grandis*) is more closely related to sesquiterpene and diterpene synthases from conifers than it is to monoterpene synthases from angiosperms (Bohlmann et al., 1997). The hop myrcene synthase exhibited 52% amino acid identity with *V. vinifera* (-)- $\alpha$ -terpineol synthase (Martin and Bohlmann, 2004), but only 29% identity to grand fir myrcene synthase. However, the hop myrcene synthase is also only 29.8% identical to myrcene synthase from snapdragon (Dudareva et al., 2003); it is more closely related (40.8%) to Arabidopsis myrcene synthase (At2g24210), although this enzyme, unlike that from hop, can also produce ocimene from GPP (Bohlmann et al., 2000).

It is well known that sesquiterpene synthases with the ability to form humulene can also form caryophyllene. Thus, rice (*Oryza sativa*) caryophyllene synthase produces caryophyllene as the major product, although several other products are made with FPP as substrate, including humulene and  $\beta$ -elemene (Cheng

et al., 2007). It is interesting that this latter product is produced by a separate enzyme (HISTS2) in hop. Similar product complexity to that observed with rice sesquiterpene synthases is seen in Arabidopsis, where genetic evidence has shown that only two enzymes account for most of the complex mixture of over 20 sesquiterpenes in the floral scent (Tholl et al., 2005). HISTS1 appears to be a less promiscuous sesquiterpene synthase than those of rice and Arabidopsis, and its dual product specificity in vitro correlates with the humulene to caryophyllene ratio in the different hop tissues in which this enzyme is expressed, suggesting that it is the major determinant of the levels of these two compounds.

Although the two monoterpene synthases described in this article have clear N-terminal plastid-targeting sequences, we could not detect similar sequences in the two sesquiterpene synthases using TargetP 1.1 software. If these latter enzymes are truly cytoplasmic and if, as we suggest, the plastidial MEP pathway is responsible for most, if not all, of the formation of the building blocks for GPP and FPP, there would need to be transport of IPP/DMAPP between plastid and cytosol in hop trichomes, as occurs in snapdragon flowers (Dudareva et al., 2005).

Although the genomics approach applied in this article successfully identified the genes involved in mono- and sesquiterpene biosynthesis in hop trichomes, the prenylation step in the formation of bitter acids and prenylflavonoids still requires elucidation. Recently, the first plant flavonoid prenyltransferase was described, an enzyme from *Sophora flavescens* that prenylates the flavanone naringenin (Sasaki et al., 2008). This enzyme is a member of the membrane-associated UbiA family of plant prenyltransferases. However, preliminary biochemical evidence suggests that the hop bitter acid prenyltransferase is a soluble enzyme (Zuurbier et al., 1998). It is not clear whether the same or different enzymes catalyze the prenylation of the different polyketide (naringenin chalcone and PIVP) intermediates in prenylflavonoid and bitter acid biosynthesis. We could only identify a single EST representing a plant UbiA family prenyltransferase in our EST collection. In contrast, 23 hop ESTs annotated as potential aromatic prenyltransferases were reported from an EST collection derived from normalized and non-normalized libraries, although none was functionally identified (Nagel et al., 2008). This suggests that the aromatic prenyltransferases of bitter acid and prenyl flavonoid biosynthesis in hop are, if similar to currently known prenyltransferases, expressed at relatively low levels in comparison to the corresponding polyketide synthases.

Bacterial prenyltransferases involved in antibiotic synthesis were identified recently and are soluble proteins that contain a conserved protein fold but share low similarity at the primary sequence level (Kuzuyama et al., 2005). The bacterial prenyltransferase sequences were also used to search against our hop unigenes, but no homologous sequences were identified.

Several previous studies have revealed a high proportion of LTP transcripts in plant trichomes (Lange et al., 2000; Aziz et al., 2005). LTPs are small basic polypeptides that bind to fatty acid derivatives and are secreted to the cell walls in plants (Kader, 1996). Some of the food allergens belong to the LTP family (Pastorello et al., 1999). The function of LTPs in trichomes is unknown, although it has been suggested that LTPs may play a role in plant defense against pathogens (García-Olmedo et al., 1998). It has also been suggested that the LTPs in peppermint glandular trichomes are involved in intracellular trafficking and secretion of essential oils (Lange et al., 2000).

In conclusion, we have described the construction and analysis of a hop glandular trichome EST database. Mining the sequences in the database resulted in the identification of many of the genes involved in terpene natural product biosynthesis, and mono- and sesquiterpene synthases responsible for formation of the major hop essential oil components were functionally characterized. The database, which is publicly available as a part of the Noble Foundation's TrichOME database (<http://trichome.noble.org/trichomedb>), provides a resource for further characterization of the molecular basis of hop trichome development and metabolism, as well as being a potential source of molecular markers to facilitate hop breeding.

## MATERIALS AND METHODS

### Plant Material

For cDNA library construction, mid-developmental stage female cones were collected from hop (*Humulus lupulus* 'Phoenix') plants, grown at the Hop Research Institute, Wye, Kent, UK. The large cones (unlikely to develop much further) and the small cones (containing few trichomes) were discarded and only the medium-sized cones were used.

Rhizomes of hops of cultivar Nugget were purchased from Northern Brewer Company and grown in the greenhouse (after flowering, the day-length was reduced from 16 to 14.5 h to initiate production of cones). Young leaves (1–2 cm in diameter), old leaves (8–10 cm in diameter), cones, and glandular trichomes (see below) were collected and stored at  $-80^{\circ}\text{C}$  until used for chemical extraction or analysis of transcript levels.

### Preparation of Trichomes

A total of 10 female cones were used for each batch of RNA isolated. The cones were frozen in liquid nitrogen and kept on ice while each bract was removed from the cone using a fine tip forceps. The bracts were transferred to a chilled 50-mL falcon tube and cold diethyl pyrocarbonate-treated water was added to submerge all the plant material. Approximately 2 g of glass beads (Sigma glass beads; 600  $\mu\text{m}$ ; acid washed) were added and the tube firmly capped. Glandular trichomes were separated by vortexing the tube for 1 to 2 min while keeping the tube in a horizontal position. Trichomes were sifted through a 500- $\mu\text{m}$  metal mesh followed by low-speed centrifugation to collect trichomes in the bottom of the tube. The trichomes were used immediately for total RNA extraction.

### RNA Isolation, cDNA Library Construction, and EST Sequencing

Total RNA was isolated from trichomes using the cold-phenol method as described (Carpenter and Simon, 1998). RNA concentration and quality were determined with a Nanodrop spectrophotometer (Thermo Fisher Scientific)

and by formamide gel electrophoresis. First-strand cDNA was synthesized from 1  $\mu$ g total RNA using a Creator Smart cDNA library construction kit following the manufacturer's protocol (CLONTECH); 16,152 colonies were randomly selected and used for inoculating liquid cultures. Plasmids were extracted from bacterial clones using Biomek 2000 robots and were submitted to single-pass 5' sequencing.

### EST Sequence Analysis and Annotation

Vector, low-quality, and short sequences (<100 bp) were subtracted from the EST database. The remaining sequences were inspected manually to further improve the quality of EST trimming. Cleansed EST sequences were used for assembling into unigenes (contigs and singletons). The parameters used for sequence assembly were minimum sequence overlap of 40 bp and minimum percentage of sequence identity of 94%.

Hop unigenes were annotated based on their best BLASTX hits in the NCBI nonredundant protein database. Unigenes with  $E < 10^{-4}$  were classified as no hit. Sequences showing high similarity to ribosomal RNA and genomic DNA were excluded from the EST database. For functional classification of the hop glandular trichome unigenes, the sequences were searched against the protein database with standard GO classifications (www.geneontology.org). The relative frequency of unigene counts assigned to each functional category was displayed in pie charts using Microsoft Excel.

### Terpene Analysis in Hop Tissues

Fresh plant material was ground to a fine powder in liquid N<sub>2</sub> using a mortar and pestle. The powder was soaked in hexane (10:1 [v/w], hexane to tissue) containing 0.03% toluene as internal standard and extracted for 2 h at room temperature in 4-mL glass vials with tightly sealed rubber septa caps. After centrifugation at 3,000 rpm for 30 min, the clear hexane layer was transferred into another vial for GC-MS analysis. For analysis of terpenes in trichomes, the hop cones were broken in liquid nitrogen and the trichomes isolated by filtration and centrifugation as described above, followed by extraction with hexane. Samples were injected at a 1:1 split ratio, and the inlet and transfer line were held at 280°C. Separation was achieved with a temperature program of 40°C for 2 min, then ramped at 10°C/min to 300°C and held for 20 min, on a 60-m DB-5MS column (J&W Scientific; 0.25 mm i.d., 0.25- $\mu$ m film thickness) with constant flow of 1.0 mL/min. Three independent biological replicates were analyzed for each data point. The myrcene, humulene, and caryophyllene contents were calculated from standard curves constructed with authentic samples. The content of the unidentified compound at retention time 10.491 min was calculated based on the standard curve of myrcene.

### Generation, Expression, and Assay of Recombinant Terpene Synthases

To obtain the N-terminal sequences of MTS1 and MTS2, 5'-RACE was performed using the MTS1-specific reverse primer 5'-TCACCCTTTGGTACAGTAGCATTGCCCC-3' and the MTS2-specific reverse primer 5'-CCG-GTCCCTCTATTGAAAGCCAAGCCATCTC-3', respectively. The open reading frames of MTS1 and MTS2 were obtained by RT-PCR using the primers 5'-ATGTCACCTTCAGGAGTGCCATTATCGGCTGG-3' and 5'-CTAAGGAG-TGGGAATTGGATTGAAAAACAAGGAG-3' for MTS1, and 5'-ATGCAGTGCATGGCTGTTACCAAITTGC-3' and 5'-TCACCTTCTCTCATGAAAAATT-AATAGGATTAATAATCAGTGC-3' for MTS2. The resulting PCR products were subcloned into pEXP5-CT/TOPO vector (Invitrogen) and the DNA sequences of MTS1 and MTS2 confirmed by sequencing at least five independent clones.

To obtain soluble proteins for expression in *Escherichia coli*, the terpene synthase inserts were truncated at the 5'-end to remove the putative N-terminal signal peptides. For MTS1 and MTS2 expression constructs, artificial open reading frames were obtained by PCR using the forward primer 5'-ATGAGGCGATCCGGCAATTACAAACC-3' and the reverse primer 5'-CTAAGGAGTGGGAATTGGATTGAAAAACAAGGAG-3' (for MTS1), and the forward primer 5'-ATGCGAAGATCAGCAACTATGAACCCTC-3' and the reverse primer 5'-TCACCTTCTCTCATGAAAAATTAATAGGATTAATAATCAGTGC-3' (for MTS2). The resulting PCR fragments were cloned into pEXP5-CT/TOPO vector following the manufacturer's instructions. Recombinant enzymes were induced by treating the cells with 0.5 mM isopropylthio- $\beta$ -galactoside overnight at 16°C.

Full-length cDNA cloning and protein expression in *E. coli* for STS1 and STS2 was performed as above, but using the following primers for STS1: 5'-GCCTCTCAAGGCTCTTCTCAATGGCCTCTC-3' for 5'-RACE and 5'-ATGTCACCTCAAATCTTAGCATCATC-3' and 5'-TCATGGAATTGGATC-AATAAGCAACCGCAGCAACACTATCTTTG-3' for production of N-terminal truncated open reading frames for protein expression in *E. coli*. Corresponding primers for STS2 were 5'-GGTGAGAAATAACAACCTTATTATTAATTTAA-CAAGC-3' for 5'-RACE and 5'-ATGTCACCTCAAATCTTTGCATCATC-3' and 5'-TCATGGAATTGGATGAATAAGCAACCGC-3' for generation of protein expression constructs.

Monoterpene and sesquiterpene synthase assays were performed as described previously (Dudareva et al., 2003; Tholl et al., 2005).

### Quantitative RT-PCR Analysis of MTS1 and MTS2 Transcript Levels

Total RNA for real-time RT-PCR analysis of terpene synthase transcripts in different tissues was isolated using the cold-phenol method after a DNA digestion step (Carpenter and Simon, 1998). Equal amounts of total RNA after treatment with the RNA Cleanup kit (Qiagen) were used for cDNA generation using Superscript III (Invitrogen) according to the manufacturer's instructions. Primer design and real-time PCR were performed by following the manufacturer's instructions. The relative amounts of transcripts for different genes were normalized to glyceraldehyde-3-P dehydrogenase (GAPDH) transcript levels using LinRegPCR software. Every PCR reaction was repeated with three independent biological replicates, each of which was represented by three technical replicates. Gene-specific primers were as follows: MTS1 forward, 5'-CTTCTCCATCCAACAAACCAACT-3', MTS1 reverse, 5'-TGC-CGGATCGCCTAACAA-3'; MTS2 forward, 5'-GGCGACGTTCTAAATC-AATTC-3', MTS2 reverse, 5'-TCACGAGCTTCGTCTTCTGAAG-3'; and GAPDH forward, 5'-TCTCCAGCTCTCAACGGTAA-3', GAPDH reverse, 5'-TGAGACATCGACGGTAGGAACA-3'.

Gene-specific primers for semiquantitative RT-PCR of HISTS1 and HISTS2 were STS1 forward, 5'-TATGGACCGCAAGATTATTAGGGCATCTTC-3', STS1 reverse, 5'-TTCTGTATTACACACTTATTATATAGAAGAGATATCC-3'; and STS2 forward, 5'-GGATATTAGCAGTCTATTATGAACCCCAATACTATT-3', STS2 reverse, 5'-GGTGAGAAATAACAACCTTATTATTAATTTAAACA-AGC-3'.

Sequence data from this article can be found in the GenBank dbEST database under accession numbers ES652314-ES658722 and EX515309-EX521564 (12,665 single-pass EST sequences), and in the GenBank gene database as accession numbers EU760348-EU760351 (HIMTS1, HIMTS2, HISTS1, and HISTS2, respectively).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Functional classification of the sequences in the hop glandular trichome cDNA library.

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