Characterization of the Formation of Branched Short-Chain Fatty Acid:CoAs for Bitter Acid Biosynthesis in Hop Glandular Trichomes

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ABSTRACT  
Bitter acids, known for their use as beer flavoring and for their diverse biological activities, are predominantly formed in hop (\textit{Humulus lupulus}) glandular trichomes. Branched short-chain acyl-CoAs (e.g. isobutyryl-CoA, isovaleryl-CoA and 2-methylbutyryl-CoA), derived from the degradation of branched-chain amino acids (BCAAs), are essential building blocks for the biosynthesis of bitter acids in hops. However, little is known regarding what components are needed to produce and maintain the pool of branched short-chain acyl-CoAs in hop trichomes. Here, we present several lines of evidence that both CoA ligases and thioesterases are likely involved in bitter acid biosynthesis. Recombinant HICL2 (carboxyl CoA ligase) protein had high specific activity for isovaleric acid as a substrate (K\textsubscript{cat}/K\textsubscript{m} = 4100 s\textsuperscript{-1} M\textsuperscript{-1}), whereas recombinant HICL4 specifically utilized isobutyric acid (K\textsubscript{cat}/K\textsubscript{m} = 1800 s\textsuperscript{-1} M\textsuperscript{-1}) and 2-methylbutyric acid (K\textsubscript{cat}/K\textsubscript{m} = 6900 s\textsuperscript{-1} M\textsuperscript{-1}) as substrates. Both HICLs, like hop \textit{valerophenone synthase} (HIVPS), were expressed strongly in glandular trichomes and localized to the cytoplasm. Co-expression of HICL2 and HICL4 with HIVPS in yeast led to significant production of acylphloroglucinols (the direct precursors for bitter acid biosynthesis), which further confirmed the biochemical function of these two HICLs \textit{in vivo}. Functional identification of a thioesterase that catalyzed the reverse reaction of CCLs in mitochondria, together with the comprehensive analysis of genes involved BCAA catabolism, supported the idea that cytosolic CoA ligases are required for linking BCAA degradation and bitter acid biosynthesis in glandular trichomes. The evolution and other possible physiological roles of branched short-chain fatty acid:CoA ligases \textit{in planta} are also discussed.

Key words: CoA ligase; glandular trichomes; bitter acid; thioesterase; evolution; \textit{Humulus lupulus}.

INTRODUCTION

Hop (\textit{Humulus lupulus} L.), which belongs to the Cannabaceae family, is an important ingredient, along with barley and yeast, for the beer-brewing industry. Three key classes of natural products are biosynthesized and stored in abundance in hop glandular trichomes: bitter acids (a mixture of humulone and lupulone derivatives) which give beer its flavor, prenylated flavonoids (primarily xanthohumol and desmethyloxanthohumol), and essential oils composed mainly of myrcene, α-humulene, and β-caryophyllene (Hirosawa \textit{et al.}, 1995; Stevens \textit{et al.}, 1998; Stevens and Page, 2004; Wang \textit{et al.}, 2008; Van Cleemput \textit{et al.}, 2009; Kavalier \textit{et al.}, 2011). Recent studies demonstrate that hop terpenophenolics (a term for both bitter acids and prenylated flavonoids) exert diverse biological activities with a high potential for drug development (Goto \textit{et al.}, 2005; Van Cleemput \textit{et al.}, 2009; Saugspier \textit{et al.}, 2012). Improvement of terpenophenolic content is thus one of the most desirable goals for hop cultivation. Additionally, hop is an economically important crop cultivated in most temperate zones of the world. The United States, after Germany, is the second largest hops producer, with an annual production value of $179.4 million in 2011 (from USDA National Hop Report 2011; \url{www.nass.usda.gov/Statistics_by_State/Washington/Publications/Hops/index.asp}). For these reasons, a combination of genomics

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approaches, reverse genetics, and in vitro biochemical assays have been employed for extensive study of terpenophenolic biosynthesis in hop trichomes, and several key enzymes had been functionally identified (Nagel et al., 2008; Wang et al., 2008; Hung and Dixon, 2009; Tsurumaru et al., 2012). Several hop trichome-specific cDNA libraries have been constructed and sequenced on a large scale in these studies. Thus far, a total of 22,959 ESTs have been deposited in a publicly available database for plant trichomes (TrichOME, www.planttrichome.org), which provides a rich transcriptional resource for further functional identification of genes involved in the terpenoplenolic biosynthetic pathways (Dai et al., 2010).

In hop, the initial step of the terpenophenolic biosynthetic pathway is catalyzed by a type III polyketide synthase such as HIVPS or chalcone synthase (CHS), which utilize different CoA-activated acids as starter units and malonyl-CoA as an extension unit (Figure 1B). Among five cloned CHS/VPS-like genes from hop, HIVPS (Genbank accession number is AB015430) exhibits high activity for the condensation reaction of isobutyryl-CoA, isovaleryl-CoA, and three molecules of malonyl-CoA to form PIBP and PIVP, respectively (Paniago et al., 1999; Okada et al., 2004). Although the polyketide synthase and downstream modification reactions have been extensively studied in many plant species (Yu and Jez, 2008), the routes to generate the CoA-activated substrates have been overlooked. It is well known that CoA-conjugated intermediates are essential components for a wide range of primary and secondary metabolic pathways in all living organisms (Shockey and Browse, 2011). Usually, carboxylate compounds are activated by CoA ligases via a two-step mechanism: the carboxylic acid group is adenylylated to form the acyl-AMP intermediate, followed by displacement of AMP with CoA to form the corresponding thioester and AMP (Figure 1A). The reverse reaction from acyl-CoA to the free acid catalyzed by thioesterases has also been characterized in plants (Tilton et al., 2004). To date, many studies have demonstrated that there is a dynamic equilibrium between the free acid and the corresponding acyl-CoA, and disruption of this equilibrium, especially by altering CoA ligase genes, causes severe growth defects in plants (Hu et al., 1999; Allen et al., 2011; Chen et al., 2011; Shockey and Browse, 2011).

The branched short-chain acyl-CoAs involved in bitter acid biosynthesis are formed via the transamination of a BCAA followed by the oxidative decarboxylation of the ketoacid intermediate, based on tracer experiments. In detail, isovaleryl-CoA is produced from leucine catabolism, 2-methylbutyryl-CoA is from the degradation of isoleucine and/or threonine, and isobutyryl-CoA is from valine catabolism (Goese et al., 1999; Karppinen et al., 2007). It is plausible to argue that, in order to provide enough BCAA for bitter acid biosynthesis, hop trichomes must have a high capacity for BCAA production, via α-keto acid elongation-mediated one-carbon-adding cycles starting from pyruvate (Kroumova et al., 1994; Binder, 2010). Consistently with this concept, unigenes involved in BCAA biosynthesis are abundant in the hop trichome EST data set (Supplemental Table 1). Additionally, it is well documented that the degradation of BCAAs occurs in mitochondria in plant cells (Binder, 2010). However, it is still unclear how these branched short-chain acyl-CoAs get to the cytosol from the mitochondria, as it is presumed they must be based on the cytosolic localization of the downstream type III polyketide synthase, and whether a CoA ligase is needed to produce and maintain the pool of branched short-chain acyl-CoA for bitter acid production in hop trichomes. Thus far, to our best knowledge, no such carboxyl CoA ligase (CCL, we use this name to designate all types of CoA ligase in this study) using branched short-chain fatty acid as a substrate has been reported from any organism.

In a previous report (Wang et al., 2008), we found one putative CoA ligase unigene assembled with 32 ESTs (out of a total of 9816 ESTs generated at the Noble Foundation prior to the addition of further ESTs to the database), which potentially could be involved in the formation of branched short-chain acyl-CoAs. To explore the role of CoA ligase in the biosynthesis of terpenophenolics in hops, we extracted unigenes encoding putative CCLs from the TrichOME database, and a total 13 full-length genes were obtained. In vitro activities for formation of isovaleryl-CoA by HICCL2, isobutyryl-CoA, and 2-methylbutyryl-CoA by HICCL4, malonyl-CoA by HICCL8, and p-coumaroyl-CoA by HICCL1 were determined. The positive correlation between the transcript levels of these HICCLs and the accumulation patterns of terpenophenolics in different tissues suggests an involvement of these CoA ligases in terpenophenolic biosynthesis in hop trichomes. Functional identification of a mitochondrial thioesterase, which hydrolyzed the branched short-chain acyl-CoA to acids, further supports the requirement for cytosolic HICCL2 and 4 in bitter acid biosynthesis in hop glandular trichomes. Our successful reconstruction of the initial steps of the bitter acid pathway by co-introducing HICCL2/HICCL4 and HIVPS in a yeast system not only provides confirmation of the in vivo activity of HICCL2 and HICCL4, but also offers a platform to characterize further the remaining enzymatic steps in the bitter acid biosynthesis pathway.

RESULTS

Bitter Acids Accumulate Mainly in Hop Glandular Trichomes

The distribution of bitter acids in different tissues of hop (cultivar Nugget) were first analyzed by HPLC/PDA/ESI–MS, and six main components were quantified (α-acids: humulone (R.T. 70.3 min), cohumulone (R.T. 67.2 min), and adhumulone (R.T. 71.1 min); β-acids: lupulone (R.T. 81.8 min), colupulone (R.T. 79.1 min), and adlupulone (R.T. 82.5 min); the β-acids differ structurally from the α-acids by containing one more dimethylallyl group). Unlike xanthohumol (R.T. 58.8 min) and desmethylxanthohumol (R.T. 53.2 min), which could also be detected in other tissues, albeit at much lower contents, bitter acids were found exclusively in mature cones and isolated
Figure 1. Proposed Roles of Carboxyl CoA Ligases in the Terpenophenolic Biosynthetic Pathway and Terpenophenolic Levels in Different Hop Tissues. (A) Proposed reactions catalyzed by carboxyl CoA ligases and thioesterases in hop glandular trichomes. Several representative acids in hop trichomes are listed.

(B) Biosynthesis of xanthohumol and bitter acids in hop glandular trichomes. BCAA, branched-chain amino acid; CHS, chalcone synthase; NC, naringenin chalcone; PIBP, phlorisobutyrophene; PIVP, phlorisovalerophene; VPS, valerophenone synthase.

(C) Quantification of terpenophenolic metabolites in different tissues of hop plants determined by an HPLC-based assay. Values represent mean ± SD. from triplicate measurements. DMX, desmethyloxanthohumol; Xan, xanthohumol; WAF, weeks after flowering.
glandular trichomes (Supplemental Figure 1). Removing the glandular trichomes from female bracts resulted in almost no detectable level of any bitter acids (Figure 1C), which suggests that the glandular trichomes are the primary site for bitter acid biosynthesis and storage. α- and β- bitter acids comprised about 199.6 and 113.1 mg g⁻¹ F.W. (n = 3), respectively, in the trichomes from 4-week-old hop cones. The precursors such as PIVP, PIIB, and naringenin chalcone accumulated in trace amounts, if at all, in the tested tissues (Supplemental Figure 1), which indicates that the next biosynthetic step, most probably catalyzed by aromatic prenyltransferase, was highly efficient and did not allow accumulation of these intermediates. These observations are mainly consistent with the previous reports (Hirosawa et al., 1995; Nagel et al., 2008).

Cloning and Sequence Analysis of CCL Genes from Hop Glandular Trichomes

We speculated that CCL could play a role in bitter acid biosynthesis, based on its high EST number in our cDNA library (Wang et al., 2008). To test this hypothesis, we extracted 38 unigenes (16 contigs and 22 singletons) representing 175 ESTs using ‘CoA ligase’, ‘acetyl-CoA synthetase’, ‘acyl-activating enzyme’ and ‘AMP-binding protein’ as key words combined with BLAST using Arabidopsis CCL genes to search the hops library on the TrichOME website. Due to our specific interest in terpenophenolic biosynthesis, unigenes annotated as ‘long-chain acyl-CoA ligase’ with high similarity (E-value < 1 e-50) and those with poor sequence quality were first removed from the candidate list (see Supplemental Table 2). Unigene GD247613 was later included in further studies due to the recent functional identification of its Arabidopsis counterpart (AAE13, At3g16170) as a malonyl-CoA synthetase (Chen et al., 2011). For all unigenes of interest, either 5’ sequence or 3’ sequence or both were missing. 5’ and 3’ RACE PCR using cDNA from isolated hop glandular trichomes as template was therefore performed, and ultimately 13 different CCL genes, designated HICCL1 through HICCL13, were obtained (Table 1; we could not acquire full-length sequence for unigene ES652801 annotated as phenylacetyl-CoA ligase for further study). All 13 CCLs contained the conserved AMP-binding motif (PS00455, Supplemental Figure 2), and they were diverse in primary sequence, with overall sequence identity ranging between 13.2% and 97.9%. Of them, HICCL2 and HICCL13 share high identity to each other (97.9%, only 12 amino acid differences between HICCL13 and HICCL2). We designed a successful RT–PCR assay to specifically amplify partial transcripts of HICCL13 and sequenced these to rule out the possibility of erroneous amplification of HICCL2 (Supplemental Figure 3).

Previous phylogenetic analysis has defined seven subfamilies of AMP-dependent synthetases and ligases in Arabidopsis (Shockey and Browse, 2011), and the 13 HICCLs fell into all clades except Clade III, containing amino-acid conjugates. Among them, HICCL2 and HICCL13 (representing 129 ESTs), accompanied by HICCL3, 4, and 11, clustered in Clade VI. The products of the Arabidopsis genes in Clade VI have not displayed any acyl-CoA synthetase activity except that AAE7 (At3g16190) and AAE11 (At1g66120) have the ability to activate C2:0/C4:0 and C5:0/C8:0 fatty acids, respectively (Shockey et al., 2003).

It is noteworthy that unigenes encoding thioesterase (24 unigenes representing 60 ESTs), which putatively hydrolyze acyl-CoAs to their corresponding acids, were also highly abundant in the hop trichome database (Supplemental Table 1).

Biochemical Characterization of HICCLs In Vitro

Although each hop CCL had a close counterpart in the Arabidopsis genome (>60% identity at the protein level, Table 1), there is currently no reliable prediction method to determine the substrate preference of enzyme from phylogenetic relationships alone due to the fact that divergent, convergent, and parallel evolution of function exists in plant specialized metabolism. Additionally, many Arabidopsis genes are still functionally uncharacterized. Therefore, we analyzed the 13 HICCLs via high-throughput activity screening of recombinant proteins (see the Methods section and Supplemental Table 3). All active hop CCLs tested in the present study exhibited high activity in the pH range 6.5–8.0, and ATP and Mg²⁺ were necessary for their CoA ligase activity in vitro. We could not obtain the HICCL11 protein in soluble form even though we tried three different expression vectors with and without fusion tags (pEXP5-CT TOPO, pMAL-c2x, and pDEST-17) and no substrates could be determined for HICCL 9 and 12, which indicated that either the endogenous substrates for these enzymes were not included in this study or they were nonfunctional proteins. All acyl-CoA products in these assays and CoASH have a specific UV absorbance at 257 nm except that coumaroyl-CoA has an additional absorbance peak at 355 nm. CCL5, 6, 7, and 10 used only medium-long-chain fatty acids (C7:0–C18:0) as substrates; thus we did not further identify their products with LC–ESI/MS. No apparent inhibitory effects of substrates (the maximum concentration of COA and acid substrate used in our assays is 0.5 and 1 mM, respectively) on any CoA ligase tested in present study were detected. The retention times and mass spectra of some enzymatic products (e.g. propionyl-CoA, isobutyryl-CoA, isovaleryl-CoA, coumaroyl-CoA, and malonyl-CoA) were identical to those of commercially available standards (Figure 2 and Supplemental Figure 4). The detailed biochemical properties of several hop CCLs that are probably involved in terpenophenolic biosynthesis are described below.

Recombinant HICCL1, as predicted based on its primary protein sequence, catalyzed the conversion of p-coumarate to coumaroyl-CoA, determined with LC–ESI/MS (Supplemental Figure 4A). HICCL1 had the highest activity towards p-coumarate (100% activity corresponds to 0.20 µmol min⁻¹ mg⁻¹ protein). Moderate activities were observed when caffeate (23%, n = 2) and ferulate (88%, n = 2) were used as substrates. HICCL8 showed high malonyl-CoA synthetase activity (0.37 µmol min⁻¹ mg⁻¹, Supplemental Figure 4F), with no
Table 1. Bioinformatic Analyses of 13 Putative Carboxyl CoA Ligases and Four Thioesterase from Hop Glandular Trichomes.

<table>
<thead>
<tr>
<th>Name</th>
<th>No. ESTs</th>
<th>BLASTP against Arabidopsis genome (functional annotation)</th>
<th>Identity (%)</th>
<th>Peptide length (amino acids)</th>
<th>Predicted subcellular localization (using Wolf PSORT and Target P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HICCL1</td>
<td>1</td>
<td>AT1G51680, 4-coumarate:CoA ligase 1</td>
<td>71</td>
<td>548</td>
<td>Plas: 6.0, Cyto: 4.0</td>
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<td>HICCL2</td>
<td>129</td>
<td>AT2G17650, function unknown</td>
<td>65</td>
<td>573</td>
<td>cTP (0.417)</td>
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<tr>
<td>HICCL3</td>
<td>6</td>
<td>AT3G16910, C2:0/C4:0 CoA ligase</td>
<td>74</td>
<td>568</td>
<td>Cyto: 5.0</td>
</tr>
<tr>
<td>HICCL4</td>
<td>4</td>
<td>AT5G16370, acyl-activating enzyme 5</td>
<td>67</td>
<td>556</td>
<td>--</td>
</tr>
<tr>
<td>HICCL5</td>
<td>4</td>
<td>AT1G20510, OPC-8:0 CoA ligase 1</td>
<td>76</td>
<td>551</td>
<td>Plas: 10.0</td>
</tr>
<tr>
<td>HICCL6</td>
<td>1</td>
<td>AT1G49430, long-chain acyl-CoA synthetase 2</td>
<td>67</td>
<td>657</td>
<td>Cyto: 6.0, Nucl: 5.0</td>
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<td>544</td>
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<td>607</td>
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<td>525</td>
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<td>563</td>
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<td>723</td>
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<td>574</td>
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<td>217</td>
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<td>437</td>
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<tr>
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<td>207</td>
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<tr>
<td>HITE4</td>
<td>4</td>
<td>AT2G30720, function unknown</td>
<td>68</td>
<td>527</td>
<td>mTP (0.596)</td>
</tr>
</tbody>
</table>

* The genetic analysis of corresponding mutants has been reported. Chlo, chloroplast; Cyto, cytoplasm; Nucl, nuclear; Mito, mitochondria; Pero, peroxisome; Plas, plasma membrane.

detectable activities towards other dicarboxylic acids (e.g. 2-methylmalonic acid, succinic acid) and fatty acids. Its close homolog, Arabidopsis AAE13, also shows low activity towards 2-methylmalonic acid in addition to its activity on malonic acid (Chen et al., 2011).

Recombinant HICCL2 showed high activity towards isovaleric acid, 3-methylvaleric acid, and 4-methylvaleric acid to produce the corresponding CoA conjugates. The structures of all enzymatic products, except isovaleryl-CoA (for which there was an authentic standard), were determined primarily based on mass spectra. In all mass spectra, no matter which ionization mode was used, the dominant ions were in good agreement with the calculated singly and doubly charged pseudo-molecular ions of the expected CoA esters (Figure 2 and Supplemental Figure 4B and 4C). HICCL2 also utilized butyric acid, valeric acid, and hexanoic acid as substrates, albeit to a lower degree (Table 2). In our in vitro biochemical assays, HICCL3 showed no apparent difference from HICCL2 in its substrate specificity; therefore, only HICCL2 was analyzed further for evaluation of steady-state kinetic parameters.

Purified HICCL3 catalyzed the formation of very-short-chain acyl-CoAs from the corresponding fatty acids, including acetic acid, propanoic acid, butyric acid, and its isomer (among them, propanoic acid and butyric acid were the two most highly preferred substrates as shown in Table 2). Weak activity was also observed with other short-chain fatty acids (Table 2). The structural confirmation of propionyl-CoA by ESI–MS/MS with authentic standards is shown in Supplemental Figure 4D.

The in vitro activity of HICCL4 was observed only towards several short-chain fatty acids (e.g. propanoic acid, butyric acid, isobutyric acid, and 2-methylbutyric acid) with the highest activities on isobutyric acid and 2-methylbutyric acid (Table 2). The mass spectra of HICCL4-catalyzed products, isobutyryl-CoA and 2-methylbutyryl-CoA (tentatively identified due to the lack of authentic standard), are shown in Figure 2D–2F and Supplemental Figure 4E, respectively.

Because isovaleryl-CoA, isobutyryl-CoA, and 2-methylbutyryl-CoA are building blocks for different types of bitter acids (humulone/lupulone, Co-humulone/lupulone), and Ad-humulone/lupulone, respectively) in hop trichomes,
we further determined the kinetic properties of HICCL2 and HICCL4 with their preferred branched short-chain fatty acids, which are known precursors for bitter acid biosynthesis. The steady-state kinetic parameters obtained for HICCL2 and 4 are summarized in Table 3. The high catalytic efficiency (Kcat/Km > 10³ M⁻¹ s⁻¹) suggested that isovaleric acid, isobutyric acid, and 2-methylbutyric acid were probably the bona fide substrates for HICCL2 and HICCL4 in hop glandular trichomes.

**Tissue-Specific/Developmental Expression Patterns and Subcellular Localization of Hop CCLs**

To test whether the transcript profiles of the above-mentioned HICCLs correlated with the pattern of bitter acid production in different tissues of hop, real-time PCR was performed using the glyceraldehyde-3-P dehydrogenase gene as an internal control. As shown in Figure 3, HICCL1, 2, 8, and 13, like HIVPS, were expressed predominately in trichomes where terpenochemics are synthesized. This observation implies that HICCL1, 2 (and its close homolog HICCL13), and HICCL8 are involved in coumarate, branched short-chain fatty acid, and malonate activation en route to terpenochemical biosynthesis in hops. HICCL4 transcript levels were highest in trichomes from 4-week-old cones, although their transcripts were also detected to some degree in other tested tissues (Figure 3).

As shown in Table 1, HICCL2 and HICCL8 were predicted to be chloroplast-localized proteins by TargetP (www.cbs.dtu.dk/services/TargetP) and WoLF PSORT (http://wolfsort.org/), but with medium scores. To test these subcellular predictions experimentally, 3SS:CCL2–GFP and 3SS:CCL8–GFP, together with 3SS:CCL4–GFP, were separately introduced into Arabidopsis leaf protoplasts. The GFP signal was detected exclusively in the cytosol for HICCL2, HICCL4, and HICCL8 GFP-fusion constructs (Figure 4). No GFP signal could be found in the chloroplasts, the red chlorophyll autofluorescence of which can be seen in Figure 4. These GFP distribution patterns were similar to those of 3SS:VPS–GFP and free GFP, which showed fluorescence patterns typical of cytosolic localization (Figure 4), suggesting that both VPS and branched short-chain fatty acid CoA ligases (e.g. CCL2 and CCL4) exert their biochemical functions in the cytosol of secretory cells in hop glandular trichomes.

**Functional Identification of Thioesterase Genes from Hop Trichomes**

It is well documented that the branched short-chain acyl-CoAs are derived from BCAA degradation in mitochondria (Goese et al., 1999). To deepen our understanding of the
Table 2. Substrate Specificity of Recombinant HICCL2, 3, 4, AAE1, and AAE2.

<table>
<thead>
<tr>
<th>Substrates</th>
<th>HICCL2</th>
<th>HICCL3</th>
<th>HICCL4</th>
<th>AAE1</th>
<th>AAE2</th>
</tr>
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<td>Acetic acid (C2)</td>
<td>N.D.</td>
<td>27</td>
<td>N.D.</td>
<td>8.7</td>
<td>4.7</td>
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<tr>
<td>Propanoic acid</td>
<td>12</td>
<td>100p</td>
<td>26</td>
<td>5.6</td>
<td>9.5</td>
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<tr>
<td>Butyric acid</td>
<td>57</td>
<td>84</td>
<td>40</td>
<td>100p</td>
<td>64</td>
</tr>
<tr>
<td>Valeric acid</td>
<td>57</td>
<td>29</td>
<td>3.4</td>
<td>77</td>
<td>50</td>
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<tr>
<td>Hexanoic acid</td>
<td>31</td>
<td>15</td>
<td>2.3</td>
<td>84</td>
<td>38</td>
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<tr>
<td>Isobutyric acid</td>
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<td>43</td>
<td>100p</td>
<td>N.D.</td>
<td>2.3</td>
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<tr>
<td>2-Methyl butyric acid</td>
<td>9.0</td>
<td>43</td>
<td>73</td>
<td>N.D.</td>
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<td>Isovaleric acid</td>
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<td>N.D.</td>
<td>12</td>
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<td>N.D.</td>
<td>2.1</td>
<td>31</td>
</tr>
<tr>
<td>4-Methyl valeric acid</td>
<td>63</td>
<td>27</td>
<td>N.D.</td>
<td>83</td>
<td>65</td>
</tr>
</tbody>
</table>

The activities were measured with substrate, ATP, and free CoASH at concentration of 0.5 mM. Data are expressed as relative mean percentages from two independent assays.

- N.D., not detectable (<2% of highest activity).
- 100% relative activity of CCL2 corresponds to 0.24 μmol min⁻¹ mg⁻¹ on isovaleric acid.
- 100% relative activity of CCL3 corresponds to 1.57 μmol min⁻¹ mg⁻¹ on propanoic acid.
- 100% relative activity of CCL4 corresponds to 0.24 μmol min⁻¹ mg⁻¹ on isobutyric acid.
- 100% relative activity of AAE1 corresponds to 0.09 μmol min⁻¹ mg⁻¹ on butyric acid.
- 100% relative activity of AAE2 corresponds to 2.43 μmol min⁻¹ mg⁻¹ on isovaleric acid.

Flux from BCAA to bitter acids, we analyzed the expression patterns of genes involved in BCAA catabolism in different tissues using qRT-PCR and found that most genes, including BCAT, those coding for BCAT, the α and β subunits of BCKD-E1 and BCKD-E2 showed a trichome-prominent pattern. We also found that the expression of IVDH, which is downstream of branched short-chain acyl-CoAs, was low and not trichome-prominent (Supplemental Figure 5).

Table 3. Kinetic Properties of Recombinant HICCL2 and HICCL4.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$K_{cat}$ (min⁻¹)</th>
<th>$K_{cat}/K_m$ (s⁻¹ M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HICCL2</td>
<td>Isovaleric acid⁵</td>
<td>69.7 ± 2.0</td>
<td>17.2 ± 0.8</td>
<td>4100</td>
</tr>
<tr>
<td></td>
<td>CoA⁵</td>
<td>110 ± 13.6</td>
<td>23.3 ± 0.78</td>
<td>3530</td>
</tr>
<tr>
<td>HICCL4</td>
<td>Isobutyric acid⁵</td>
<td>164 ± 26.5</td>
<td>18.3 ± 1.4</td>
<td>1800</td>
</tr>
<tr>
<td></td>
<td>2-methyl butyric acid⁵</td>
<td>25.2 ± 2.8</td>
<td>10.5 ± 0.2</td>
<td>6900</td>
</tr>
<tr>
<td></td>
<td>CoA⁵</td>
<td>356 ± 109</td>
<td>167 ± 35</td>
<td>7818</td>
</tr>
</tbody>
</table>

- Data are presented as mean ± SD from triplicate independent assays.
- Kinetic parameters were determined with 0.5 mM CoA.
- Kinetic parameters were determined with 1 mM isovaleric acid.
- Kinetic parameters were determined with 1 mM isobutyric acid.

The apparent different subcellular localizations of BCAA degradation (in mitochondria) and CCLs/VSPs (in the cytosol) raised the question of how these branched short-chain acyl-CoAs generated from BCAA degradation in mitochondria get to cytosol for bitter acid production. One possibility is that acyl-CoAs are transported to the cytosol via a transporter protein, like the Arabidopsis COMATOSE (At4g39850; Footitt et al., 2002), a functionally identified acyl-CoA transporter. However, no COMATOSE homologs could be found in the hop trichome EST database, making this hypothesis unlikely. Meanwhile, high numbers of ESTs corresponding to thioesterase in the data set support another hypothesis: the CoA esters could be hydrolyzed to the corresponding acids by a thioesterase in mitochondria and then exported to the cytosol by diffusion or via a carrier protein, and CCL2 and 4 could be required for re-esterification to provide adequate CoA ester substrates for bitter acid formation in the cytosol. To test this hypothesis, we cloned the full-length cDNA of four thioesterase unigenes with high EST counts (designated as HITE1 to HITE4 based on their EST counts from high to low) for further function identification (Table 1 and Supplemental Table 1). Among these four putative hotdog-fold thioesterases, HITE1 and HITE3 each contained one hotdog fold and showed high identity to SIMKS2, which was recently demonstrated to hydrolyze 3-ketoacyl-acyl carrier proteins to liberate 3-ketoacids (Supplemental Figure 6; Yu et al., 2010). HITE2 and HITE4, each containing two hotdog folds and sharing 68.5% amino-acid identity, were highly similar to functionally uncharacterized thioesterases from plant species ranging from green algae to higher plants (Supplemental Figure 7).

All four HITEs showed trichome-prominent expression profiles (Figure 5A), which did not provide us with enough information to determine which TE contributed most to bitter acid biosynthesis. Thus, all four TEs were heterogeneously expressed in Escherichia coli and purified. We could not obtain the HITE2 protein in a soluble form for further biochemical investigation. In vitro enzymatic assays, however, clearly showed that the recombinant HITE4 specifically hydrolyzed acyl-CoAs with short chains (propionyl-CoA and butyryl-CoA), branched short chains (isobutyryl-CoA, 2-methylbutyryl-CoA, isovaleryl-CoA), and medium chains (octanoyl-CoA), and exhibited low activities towards long-chain acyl-CoAs (lauroyl-CoA) and no activity towards acetyl-CoA, malonyl-CoA, and palmitoyl-CoA (Supplemental Figure 8). HITE4 showed much higher catalytic efficiency ($K_{cat}/K_m$ ratio) towards isobutyryl-CoA and isovaleryl-CoA (2296 and 3915 M⁻¹ s⁻¹, respectively) than other CoA esters, for which the $K_{cat}/K_m$ could not be calculated due to the too high a $K_m$-value (>10 mM; Table 4). No hydrolytic activity against any tested acyl-CoA was detected for recombinant HITE1 and HITE3.

To test the subcellular localization of HITE4, a construct in which the first 70 amino acids of HITE4 were fused with GFP (35S:TE4SP–GFP) was introduced into Arabidopsis leaf protoplasts. Perfect co-localization of the TE4SP–GFP with
Figure 3. qRT–PCR Analyses of Nine HICCLs in Different Tissues and at Different Development Stages of Cones and Trichomes. Transcript levels are expressed relative to that of GAPDH (n = 3; mean ± SD).

MitoTracker Red, a marker dye specific for mitochondria, was observed (Figure 5B). These results were also consistent with the computational prediction by Target P and Wolf PSORT (Table 1).

Heterologous Expression and Functional Characterization of HICCL2 and HICCL4

Although there have been sporadic reports of successful hop transformation (Horlemann et al., 2003; Batista et al., 2008), the failure to establish a reliable hop transformation system, including transient gene-silencing techniques, prevented us from testing the biological functions of HICCL2 and HICCL4 in hop plants. To verify whether HICCLs have the same catalytic activities in vivo as observed in vitro, we examined the activity of HICCL2 and HICCL4 in an engineered yeast strain harboring hop HIVPS by monitoring the production of PIVP/PIBP, which is more reliable and reproducible than the measurement of acyl-CoA in vivo. For this purpose, we first over-expressed HIVPS driven by the GAL1 promoter in yeast cells. However, we found only trace amounts of PIVP/PIBP in transformant yeast either in the medium or in the cells (the highest production of PIVP was 0.1 ± 0.006 μM; n = 3), which suggests that the endogenous content of branched short-chain acyl-CoA is very low in yeast and that this was probably the main barrier to the production of PIVP/PIBP/PIMBP. Genome searches revealed that yeast did not have close homologs of HICCL2 (CH408048.1, the closest homolog annotated as...
Table 4. Kinetic Properties of Recombinant HITE4 with Different acyl-CoAs.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>(K_m) (µM)</th>
<th>(K_{cat}) (s(^{-1}))</th>
<th>(V_{max}/K_m) (s(^{-1}) M(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HITE4</td>
<td>Propionyl-CoA &gt; 10 000</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Butyryl-CoA &gt; 10 000</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isovaleryl-CoA 979.6 ± 25.5</td>
<td>135 ± 18.6</td>
<td>2296</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isovaleryl-CoA 513.3 ± 18.7</td>
<td>120.6 ± 1.74</td>
<td>3915</td>
<td></td>
</tr>
<tr>
<td>Octanoyl-CoA &gt; 10 000</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a Data are presented as mean ± SD from triplicate independent assays.}\)

The combination of HICL4 and HIVPS in yeast led to higher PIPB (2.83 ± 0.27 µM, \(n = 3\)) and PMBP production (1.50 ± 0.15 µM, \(n = 3\)) compared to the strain expressing HIVPS alone with exogenous valine stimulation (Figure 6B, 6D, and 6E). However, although leucine addition had a clear positive effect on PIVP production, it exerted an inhibitory effect on PIPB production (inhibition ranged from 50% to 80% in different genotype backgrounds) (Figure 6D and 6E). The reasons for this are not clear at present.

**Phylogenetic Analysis and Functional Identification of HICCL2 Homologs**

An unrooted phylogenetic tree was constructed from 65 amino acid sequences of CCL2 homologs from various plant species to explore the evolution and biological roles of HICCL2 homologs in planta. Based on the CoA ligase classification in *Arabidopsis*, all of these selected proteins belong to Clade VI (Shockey and Browse, 2011). The phylogenetic analysis showed that HICCL2 homologs further cluster into four distinct subclades, herein designated as Clades Vla to Vld, and HICCL2, 3, and 4 fell into Clades Vla, Vlb, and Vlc, respectively (Figure 7). The biochemical characterization of HICCL2−4 shed light on the potential functions of the homologs from other plant species. However, little biochemical information was available to suggest a biological function of HICCL2 homologs in vivo. To fill this gap, we prepared N-terminal

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**Figure 5. Expression Profiling of Different HITEs in Hop and the Subcellular Localization of HITE4.**

(A) Tissue-specific expression of HITE1–4 by qRT–PCR analyses. Transcript levels are expressed relative to those of GAPDH (\(n = 3\); mean ± SD).

(B) Subcellular localization of HITE4 in *Arabidopsis* leaf-mesophyll protoplasts. Mitochondria are revealed by MitoTracker® Red-staining. Scale bar = 5 µm.
6 X His-tag fusion proteins of AAE1 (At1g20560) and AAE2 (At2g17650), the closest homolog of HICCL2 in Arabidopsis. Biochemical assays showed that AAE2 preferred isovaleric acid as a substrate, whereas AAE1 showed high activities towards C4–C6 straight chain fatty acids (Table 2). Interestingly, most of the closest homologs of HICCL2 in Clade VIa had a predicted mitochondrial signal peptide (Figure 7). We also used computational methods to gain subcellular localization predictions for other selected genes (Wolf PSORT with a reliable score >7.0). The results showed that the independent appearance of duplicate gene pairs with different subcellular localizations occurred frequently in many plant lineages (Figure 7).

**DISCUSSION**

In this study, we used a reverse functional genomics approach to characterize the functions of 13 hop CCLs and discovered HICCL2 and HICCL4 with unprecedented branched short-chain fatty acid:CoA ligase activity, which is involved in bitter acid biosynthesis. This represents the first successful demonstration of CoA ligases specifically recognizing branched short-chain fatty acids as substrates in any organism.

**HICCL2 and HICCL4 Are Highly Specific Branched Short-Chain Fatty Acid:CoA Ligases**

Plant glandular trichomes, which exclusively synthesize and store species-specific chemicals, have proven to be excellent systems for dissection of the biosynthesis of specialized plant metabolites (Schilmiller et al., 2008). The high abundance of certain transcripts in glandular trichome databases points to them as candidate genes for involvement in the biosynthetic pathways of interest based on the ‘guilt by association’ principle (Saito et al., 2008). The high abundance of HICCL2 transcripts in our hop glandular trichome EST library (assembled with 32 ESTs and ranked 13th in the top 15 most abundant unigenes) led us to test the possible role of CoA ligase in bitter acid biosynthesis (Wang et al., 2008). Here we present multiple lines of evidence to support that HICCL2 and HICCL4 play a key role in bitter acid biosynthesis: (1) the
Figure 7. Phylogenetic Analyses of HICCL2-4 and Their Homologs from Other Plant Lineages.

All proteins used for tree construction were extracted from the PHYTOZOME 7.0 site (www.phytozome.net). Sequences were aligned using ClustalW and tree construction was generated by the neighbor-joining method using MEGA5.05 (Tamura et al., 2011). Bootstrap values in percentage (>70%), calculated with 1000 replicates, are indicated on the nodes. The preferred substrates are listed to the right of the gene name if the biochemical data are available. The sequences with a clear mitochondrial localization peptide are marked with open diamonds, peroxisomal localization is indicated by an open circle, chloroplastic localization by a solid circle, and cytosolic localization by a solid diamond. The instances of altered subcellular localization after gene duplication are highlighted with open boxes, and the four hop CCLs identified in this study are marked with arrows. Ac, Aquilegia coerulea; BA, benzoate; Mgu, Mimulus guttatus; Mtr, Medicago truncatula; Pine, Pinus sylvestris; Ppa, Physcomitrella patens; Smo, Selaginella moellendorffii; Vca, Volvox carteri; Vvi, Vitis vinifera.
high transcription levels of HICL2 and HICL4 in trichomes revealed by real-time PCR are consistent with bitter acid accumulation patterns and that of HIVPS (Figures 1 and 3); and (2) biochemical analyses of HICL2 and HICL4 clearly showed that HICL2 could utilize isovaleryl-CoA to form isovaleryl-CoA with high efficiency ($K_{cat}/K_m$ of 4100 s$^{-1}$ M$^{-1}$), whereas HICL4 could efficiently utilize the isobutyric acid and 2-methylbutyric acid ($K_{cat}/K_m$ values of 1800 and 6900 s$^{-1}$ M$^{-1}$, respectively); and (3) the most important direct evidence was obtained from the partial reconstruction of bitter acid biosynthesis in yeast cells, co-expression of HICL2/HIVPS or HICL4/HIVPS resulted in high production of PIVP or PIBP/PMBP, whereas HIVPS alone could not support similar production due to the lack of branched short-chain fatty acid CoA esters; and (4) functional identification of HITE4, which catalyzed the reverse reaction of CCL2/4 in mitochondria, together with the low level of IVDH in BCAA catabolism, support the requirement of HICL2 and 4 for reactivation of branched short-chain fatty acids in the cytosol of secretory cells in glandular trichomes.

Interestingly, although both HICL2 and HICL4 showed high activities towards branched short-chain fatty acids, they had distinct differences in their substrate specificity. HICL4 showed a substrate preference for those branched short-chain fatty acids with a methyl group at the C2 position (e.g. isobutyric acid and 2-methylbutyric acid; Figure 2B and Supplemental Figure 4E), whereas HICL2 had high activity towards those branched short-chain fatty acids with the methyl group further away from the carboxyl group (e.g. isovaleric acid, 3-methylvaleric acid, and 4-methylvaleric acid). Determining the 3-D crystal structures of these enzymes bound to their preferred substrates would deepen our understanding of the reaction mechanisms and substrate specificities of HICL2 and 4.

The Formation of Branched Short-Chain Acyl-CoAs for Bitter Acid Biosynthesis Occurs in the Cytosol

In plants, branched short-chain acyl-CoAs originate from the BCAA degradation that occurs in mitochondria (Goese et al., 1999; Binder, 2010) and the generated CoA esters, in theory, cannot cross the membrane due to their high polarity. Thus, the cytosolic localization of HIVPS (Figure 4) raised the question of how HIVPS could access its substrates, such as isobutyryl-, isovaleryl-, 2-methylbutyryl-CoA, and malonyl-CoA, to produce PIBP, PIVP and PMBP. A comprehensive analysis of the hop trichome EST data set (Supplemental Table 1) revealed a thioesterase probably involved in the release of branched short-chain fatty acids from mitochondria to the cytosol. From four candidate genes encoding thioesterase, we functionally identified one mitochondrial thioesterase (HITE4) that hydrolyzed branched short-chain acyl-CoA to acids (Supplemental Figure 8 and Table 4). This observation is also supported by the fact that labeled isovaleric acid is detected from isolated Arabidopsis mitochondrial extracts when incubated with [U-14C] leucine (Anderson et al., 1998).

The mitochondrial localization of HITE4 further confirms the requirement for HICL2 and 4 in the cytosol of secretory cells, in which these free branched short-chain fatty acids could be re-esterified by HICL2 and 4 for bitter acid formation. Based on the experimental data, the proposed pathway from BCAs to bitter acids in hop glandular trichomes is summarized in Figure 8. In this proposed pathway, it is plausible that IVDH would compete with HITE4 for consumption of branched short-chain acyl-CoAs. qRT–PCR analysis clearly showed that most genes involved in BCAA catabolism (including BCAT, and the α and β subunit of BCKD-E1, BCKD-E2) display a trichome-prominent expression pattern. However, the low level of IVDH, combined with high trichome-specific HITE4, would drive carbon flux to bitter acid production (Figure 5 and

![Figure 8. Schematic of Proposed Biosynthetic Pathway for Bitter Acids in Hop Glandular Trichomes.](https://example.com/figure8.png)

High trichome-specific expression of BCAT, BCKDH, TE, CCL, VPS, and PT drive the carbon flux from BCAA degradation towards bitter acid production. Solid arrows depict steps supported by present study; dashed arrows represent proposed steps remaining to be elucidated. BCAT, branched-chain amino-acid aminotransferase; BCKDH, branched-chain keto acid dehydrogenase; CCL, carboxyl CoA ligase; DMAPP, dimethylallyl diphosphate; IVDH, isovaleryl-CoA-dehydrogenase; MCC, methylcrotonyl-CoA carboxylase; PTase, prenyltransferase; TE, thioesterase; VPS, valero-phenone synthase.
Supplemental Figure 5). In theory, an additional advantage of the proposed pathway is that the released CoASH from the acyl-CoA by TDE could be immediately recycled by the branched-chain keto acid dehydrogenase complex for BCAA degradation, which would increase the BCAA degradation efficiency. As a critical intermediate of the TCA cycle and many other metabolic pathways, high CoASH levels are required for optimal mitochondrial function. The pool of free CoASH inside the mitochondria would be depleted rapidly, leading to increased risk of deleterious effects on mitochondrial function, if the branched short-chain acyl-CoA were continually exported outward for bitter acid formation in glandular trichomes.

Unlike that in plant cells, BCAA biosynthesis occurs in mitochondria in yeast cells, and there are at least three independent catabolic pathways, mainly operated in the cytosol, for BCAA degradation (Dickinson et al., 2000). In spite of the origin of BCAA degradation, isovaleric acid, isobutyric acid, and 2-methylbutyric acid could be detected in yeast cells, which makes it theoretically possible to test the functions of HICL2 and HICL4 in vivo (Lilly et al., 2006). Our results clearly showed that, in yeast, the availability of branched short-chain acyl-CoAs was the limiting factor for PIVP/PBIP production. Feeding the different engineered yeast strains with BCAA also supported this notion: the CoA ester substrates for PIVP and PBIP production are generated from leucine and valine degradation, respectively, and could be maintained by the actions of HICL2 and HICL4 in the engineered yeast. It is possible that the same is true of bitter acid biosynthesis in hop glandular trichomes, which is reflected by the high levels of trichome-specific HICL2 and HICL4 transcripts. The decrease in leucine and valine content with cone development of Willamette hops, which shows a clear reverse trend relative to the bitter acid accumulation pattern, is consistent with the notion that the side chains of PIVP/PBIP arise from the degradation of BCAs (Kavalier et al., 2011).

**The Evolution and Other Possible Physiological Functions of HICL2 Homologs in Plants**

Our comprehensive phylogenetic analysis shows that the Clade VI subfamily expanded rapidly after divergence from bryophytes (e.g. Physcomitrella patens), mainly via segmental and tandem gene duplication (Figure 7, combined with the analysis done by Shockey and Browse (2011)). Thus far, all functionally identified CCL2 homologs, except Arabidopsis BZ1 (At1g65880), which was recently found to use cinnamate as a substrate (Lee et al., 2012), show comparable activities towards short-chain fatty acids and no activity towards long-chain fatty acids (Figure 7). This suggests that the ancestral gene product might have possessed CoA ligase activity towards short-chain fatty acids (most probably an ancestral acetyl-CoA synthetase). Certainly, this conclusion needs to be confirmed by biochemical characterization of more Clade VI genes from different plant species, especially those in charophytes and bryophytes. It is generally accepted that the retention of both duplicated genes in a genome structure requires at least partial divergence in biological function (Thomas et al., 2006). Thus, the frequent occurrences of altered subcellular localization after gene duplication in the Clade VI subfamily could be an explanation for the quick expansion of this gene subfamily over plant evolutionary time (the duplicate genes with different subcellular localization are boxed in Figure 7). A similar case of a cytoplasm-localized hexanoyl-CoA synthetase (CsAAET1) and its tandemly duplicated homolog (CsAAET12, which possesses a functional peroxisome targeting signal peptide) for cannabinoid biosynthesis has recently been reported in Cannabis sativa trichomes (Stout et al., 2012). Certainly, other traits of duplicate genes, such as different spatiotemporal expression and different enzymatic properties, also likely contribute to the Clade VI subfamily expansion.

The predicted mitochondrial localization of Arabidopsis AAE2 and its preference for isovaleric acid as a substrate are consistent with the occurrence of leucine degradation in plant cells (Table 2) (Binder, 2010), which suggests that AAE2 might play a role between leucine degradation and the electron transport chain of Arabidopsis mitochondria in extended darkness (Araujo et al., 2010). The biological function of those AAE2 close homologs with mitochondrial signal peptides could be reasonably deduced from this notion (Figure 7). Branched short-chain acyl-CoAs are the major building blocks for branched-chain ester formation, branched-chain fatty acid elongation, acylation reactions catalyzed by BAHD acyltransferases, and polyketide biosynthesis (Kroumovova et al., 1994; Graham and Eastmond, 2002; Kroumovova and Wagner, 2003): type III polyketide synthases use branched short-chain acyl-CoA as a starter unit, such as during bitter acid biosynthesis in hops and the formation of hyperforin (a kind of prenylated phloroglucinol with a similar structure to bitter acids) in Hypericum perforatum (Karpinnen et al., 2007). Logically, at least one gene encoding a branched short-chain fatty acid:CoA ligase is expected to participate in the formation of hyperforin in St John’s wort.

Collectively, our data provide evidence that branched short-chain fatty acid:CoA ligases of different plant origins are probably involved in different physiological processes even though they might have conserved biochemical activity. In hop glandular trichomes, cytosolic CCLs, together with mitochondrial TEs, play a key role in linking BCAA degradation and bitter acid biosynthesis. Although further work, including gain- or loss-of-function experiments in hop plants, is required to determine the precise physiological roles of the genes cloned in the present study, the functional identification of a set of branched short-chain fatty acid:CoA ligases in the present study lays a solid foundation for metabolic engineering of these high-value plant metabolites (Facchini et al., 2012), as well as providing potential for hop molecular marker-assisted breeding.
METHODS

Plant Materials, RNA Analysis, and Chemical Profiling by HPLC-PDA-MS

The growth of *Humulus lupulus* cv. Nugget, cDNA preparation from hop tissue RNA, and quantitative real-time PCR were performed as described previously (Wang et al., 2008). All commercial chemicals were purchased from Sigma-Aldrich except naringenin chalcone, xanthohumol, desmethylnxanthohumol, and colupulone, which were obtained from Apin Chemicals Limited (Oxon, United Kingdom).

The different tissues of hop (50 mg fresh weight) were extracted with 0.5 ml 80% CH₃OH in H₂O and the resulting extract was analyzed with an Agilent HP1100 HPLC/PDA/ESI/MS coupled with a C18 reverse phase column (Φ4.6 mm x 250 mm, 5 μm; J.T. Baker, Phillipsburg, NJ). The elution program for LC and the parameters of the mass analyzer were set as described previously (Huhman et al., 2005). The identification of terpenophenolics was carried out by comparison of the retention time and mass spectrum with those of authentic standards or in the literature (Zhang et al., 2004). The xanthohumol and desmethylnxanthohumol contents were calculated from a standard curve of authentic xanthohumol (370 nm; R.T. 58.8 min), whereas contents of all six different bitter acids were calculated based on the standard curve of authentic colupulone (peak area obtained at 310 nm).

Three independent biological replicates were carried out for each data point of real-time PCR and chemical separation/quantification.

Expression, Purification, and Assay of Recombinant CCLs and Thioesterase Genes from Hop and Arabidopsis

To obtain the full-length sequences of 13 CoA ligases from hops, 5' and 3' RACE were performed with the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) following the manufacturer’s instructions. The open reading frames of *HIC1L*, *HITE1*, and *Arabidopsis AAE1* and 2 obtained by RT–PCR were subcloned into pEXPS-CT TOPO vector (Invitrogen) and verified by sequencing of five to eight independent clones (see Supplemental Table 4 for primer information). The expression of recombinant protein in an *E. coli* system and purification using His-tag affinity columns were performed as described previously (Wang and Dixon, 2009). The *HITE4* gene was N-terminally truncated to aid solubility. The relative purity of recombinant proteins was checked with SDS–PAGE.

We performed a rapid and high-throughput biochemical assay for the initial screening of different CoA ligases. All potential substrates were collected and pooled into six groups: Group 1, short-chain fatty acids; Group 2, branched short-chain fatty acids; Group 3, medium-long chain fatty acids; Group 4, dicarboxylic acids; Group 5, phenylpropionates; and Group 6, aromatic acids. Each group of stock solutions contained five to eight compounds at 50 mM final concentration.

(see Supplemental Table 3). Usually, an enzymatic reaction (200 μl volume with around 2 μg purified recombinant protein) was incubated at 30°C for 12 min, and the decrease in NADH content was monitored every minute at a fixed wavelength of 340 nm (ε₂₄₀ nm = 6220 M⁻¹ cm⁻¹ while NADH has practically no absorbance at this wavelength) with a Microplate Reader in 96-well plates (Infinite® 200 PRO, TECAN, Austria). Control reactions were performed with the boiled purified enzyme. The activities of CCLs were calculated using linear regression of 10–12 measured data points with R² > 0.95. The 4-coumarate CoA ligase assays for *HIC1L* were performed following the protocol described by Lee et al. (1997). Kinetic data were calculated using Hanes plot (Hyper32, version 1.0.0).

To identify the products of hop CCL1, 2, 3, 4, and 8, the assay sample (scaled up to 500 μl and 30 min of incubation) was loaded on UHPLC (Agilent, 1290 Infinity) coupled with a C18 column (ZORBAX Eclipse Plus C18, Φ2.1 x 50 mm, 1.8 μm) and mass analyzer (Agilent, 6490 triple quadrupole mass system) after being filtered through a 0.22-μm filter. The gradient (solvent A, 0.1% formic acid in water; solvent B, HPLC grade acetonitrile) program was set as follows at a flow of 0.3 ml min⁻¹: 0–0.5 min, 2% of solvent B; 0.5–3 min, a linear gradient to 60% of B; 3–3.5 min, a linear gradient to 90% of B; 3.5–4.5 min, 90% of B; 4.5–4.6 min, a linear gradient to 2% of B; 4.6–6 min, 2% of B. The mass data were collected from 200–1000 m/z with a 100-ms scan time using both positive and negative modes. The ionization source conditions were: capillary voltage: 3 kV for positive mode and 4 kV for negative mode; cone voltage: 30 V; drying gas flow: 12 L min⁻¹; 280°C; nebulizer pressure: 20 psi; nozzle voltage: 1.5 kV for both positive and negative modes. Data acquisition and evaluation were carried out with the Masshunter Workstation software package (Version B 04.01; Agilent).

The thioesterase activity (100 μl, including 2 μg purified protein, appropriate concentration of different acyl-CoAs, 0.5 mM 5′-5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and 20 mM NaH₂PO₄, pH 7.0) was monitored at 412-nm wavelength minutely using a Microplate Reader for the absorbance of the released thionitrobenzoic acid in the reaction (ε₄₁₂ nm = 14 150 M⁻¹ cm⁻¹).

Subcellular Localization of Hop CCLs and Thioesterases

Constructs for GFP-fusion proteins (pJIT163–hGFP vector), *Arabidopsis* leaf protoplast preparation, transformation, and image collection using laser scanning confocal microscopy were performed as described previously (Yoo et al., 2007).

Partial Reconstruction of Bitter Acid Biosynthesis in Yeast and Product Analysis

*HIVPS* was subcloned into the pESC–His vector, and different *HIC1L* genes were subcloned into the pESC–Leu vector. Two constructs (either empty vector or the construct with insert) were co-transformed into yeast strain YPH499 using a high-efficiency lithium acetate transformation protocol (Gietz
and Woods, 2002). The resulting positive yeast clones, after verification by PCR, were cultured for 2 d in 30 ml SD drop-
out medium (-Leu, -His) with D-glucose as the carbon source.
Then, the yeast cells were harvested and re-suspended in 5G dropout medium (-Leu, -His) with 2% galactose, and
different BCAAs were added to a final concentration of 2mM. After induction for different periods, 5-ml aliquots of
cultures, separated into yeast cells (broken by sonication before extraction) and medium after measurement of the
cell density, were extracted with an equal volume of ethyl acetate (10 μM naringenin was added as internal standard).
Extracts were dried under nitrogen stream and sub-
sequently dissolved in 40% methanol for chemical analysis
by LC–MS using MRM (multiple reaction monitoring) mode
(Naringenin, 273→153, CE = 18V; PIVP, 211→155, CE = 20V;
PIBP, 197→179, CE = 15V; PMBP, 211→123, CE = 20V). The
gradient (solvent A, 0.05% formic acid in 10% acetonitrile; solvent B, 0.05% formic acid in 90% acetonitrile) and both sol-
vents A and B contain 5mM ammonium formate) program was
set as follows at a flow of 0.35 ml min⁻¹: 0–0.5 min, a linear
gradient from 0 to 40% of B; 0.5–2.5 min, 40% of B; 2.5–
3.5 min, a linear gradient to 100% of B; 3.5–4.5 min, 100% of
B; 4.5–4.51 min, a linear gradient to 100% of A; 4.51–6 min,
100% of A. The PIVP/PIBP contents were quantified based
on the standard curve of authentic chemicals.

SUPPLEMENTARY DATA

Supplementary Data are available at Molecular Plant Online.

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REFERENCES

Allen, E., Moing, A., Wattis, J.A., Larson, T., Maucourt, M.,
ACN1 (acetate non-utilizing 1) prevents carbon leakage from
peroxisomes during lipid mobilization in Arabidopsis seedlings.

Anderson, M.D., Che, P., Song, J.P., Nikolaou, B.J., and Wurtzele, E.S.
(1998). 3-Methylcrotonyl coenzyme A carboxylase is a compo-
nent of the mitochondrial leucine catabolic pathway in plants.
Plant Physiol. 118, 1127–1138.

Araujo, W.L., Ishizaki, K., Nunes-Nesi, A., Larson, T.R., Tohge, T.,
(2010). Identification of the 2-hydroxyglutarate and isovaleryl-
CoA dehydrogenases as alternative electron donors linking
lysine catabolism to the electron transport chain of Arabidopsis

Batista, D., Fonseca, S., Serrazina, S., Figueiredo, A., and Pais, M.S.
(2008). Efficient and stable transformation of hop (Humulus
27, 1185–1196.

Binder, S. (2010). Branched-chain amino acid metabolism in
Arabidopsis thaliana. The Arabidopsis Book/American Society
of Plant Biologists. 8, e0137.

synthetase, encoded by ACYL ACTIVATING ENZYME13, is essen-
tial for growth and development of Arabidopsis. Plant Cell. 23,
2247–2262.

Dai, X., Wang, G., Yang, D.S., Tang, Y., Broun, P., Marks, M.D.,

(2000). An investigation of the metabolism of isoleucine to
active amyl alcohol in Saccharomyces cerevisiae. J. Biol. Chem.
275, 10937–10942.

Facchin, P.J., Bohlmann, J., Covello, P.S., De Luca, V., Mahadevan,
R., Page, J.E., Ro, D.K., Sensen, C.W., Storms, R., and Martin,
V.J.J. (2012). Synthetic biosystems for the production of high-

Footitt, S., Slocombe, S.P., Larner, V., Kurup, S., Wu, Y.S., Larson, T.,
mination and lipid mobilization by COMATOSE, the Arabidopsis
homologue of human ALDP. EMBO J. 21, 2912–2922.

lithium acetate/single-stranded carrier DNA/polychydroxy gly-
col method. Guide to Yeast Genetics and Molecular and Cell
Biology, Pt B. 350, 87–96.

Goese, M., Kammhuber, K., Bacher, A., Zenk, M.H., and Eisenreich,
W. (1999). Biosynthesis of bitter acids in hops: a C-13-NMR and
H-2-NMR study on the building blocks of humulone. Eur.
J. Biochem. 263, 447–454.

Goto, K., Asai, T., Haru, S., Namatame, I., Tomoda, H., Ikemoto, M.,
and Oku, N. (2005). Enhanced antitumor activity of xanthohu-
mol, a diacylglycerol acyltransferase inhibitor, under hypoxia.

Graham, I.A., and Eastmond, P.J. (2002). Pathways of straight and
branched chain fatty acid catabolism in higher plants. Progress
in Lipid Research. 41, 156–181.

observation and HPLC analysis of the accumulation of alpha-
alpha and beta-acids in the fresh developing hop (Humulus


Thomas, B.C., Pedersen, B., and Freling, M. (2006). Following tetraploidy in an Arabidopsis ancestor, genes were removed preferentially from one homeolog leaving clusters enriched in dose-sensitive genes. Genome Res. 16, 934–946.


