Noncatalytic chalcone isomerase-fold proteins in Humulus lupulus are auxiliary components in prenylated flavonoid biosynthesis

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Noncatalytic chalcone isomerase-fold proteins in *Humulus lupulus* are auxiliary components in prenylated flavonoid biosynthesis

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Contributed by Richard A. Dixon, April 25, 2018 (sent for review February 6, 2018; reviewed by Joerg Bohlmann and Mattheos A. G. Koffas)

Xanthohumol (XN) and demethylxanthohumol (DMX) are specialized prenylated chalconoids with multiple pharmaceutical applications that accumulate to high levels in the glandular trichomes of hops (*Humulus lupulus* L.). Although all structural enzymes in the XN pathway have been functionally identified, biochemical mechanisms underlying highly efficient production of XN have not been fully resolved. In this study, we characterized two noncatalytic chalcone isomerase (CHI)-like proteins (designated as HlCHIL1 and HlCHIL2) using engineered yeast harboring all genes required for DMX production. HlCHIL2 increased DMX production by 2.3-fold, whereas HlCHIL1 significantly decreased DMX production by 30%. We show that CHIL2 is part of an active DMX biosynthetic metabolon in hop glandular trichomes that encompasses a chalcone synthase (CHS) and a membrane-bound prenyltransferase, and that IV CHI-fold proteins of representative land plants contain conserved function to bind with CHS and enhance its activity. Binding assays and structural docking uncover a function of HlCHIL1 to bind DMX and naringenin chalcone to stabilize the ring-open configuration of these chalconoids. This study reveals the role of two HlCHILs in DMX biosynthesis in hops, and provides insight into their evolutionary development from the ancestral fatty acid-binding CHI-fold proteins to specialized auxiliary proteins supporting flavonoid biosynthesis in plants.

*H. lupulus* | trichome

**Significance**

Here, we identify two noncatalytic chalcone isomerase-fold proteins, which are critical for high-efficiency prenylchalcone production in *Humulus lupulus*. Our results provide insights into their evolutionary development from the ancestral noncatalytic fatty acid-binding chalcone isomerase-fold proteins to specialized auxiliary proteins supporting flavonoid biosynthesis in plants, and open up the possibility of producing high-value plant prenylchalcones using heterologous systems.

Author contributions: R.A.D. and G.W. designed research; Z.B., A.J.M., and B.L. performed research; H.Q. and F.Z. contributed new reagents/analytic tools; Z.B., J.-K.W., and G.W. analyzed data; and Z.B. and G.W. wrote the paper.

Reviewers: J.B., University of British Columbia; and M.A.G.K., Rensselaer Polytechnic Institute.

The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. HlCHIL1, HlCHIL2, MG324004, and MG324005).

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gene, CHS H1, has been identified from hops (24). NC is then prenylated by HIPT1L, and further methylated by an O-methyltransferase (HIOMT1) to form XN (20, 23, 25). Paradoxically, previous transcriptome data had indicated that several chalcone isomerase (CHI; EC 5.5.1.6) genes represent the most abundant ESTs from hop glandular trichomes that accumulate massive amounts of chalcone (1, 21). While bona fide CHI enzymes were the first to be identified in the context of plant flavonoid metabolism, CHI-fold family proteins are more widespread in other domains of life, such as fungi and bacteria (26). The plant CHI family can be classified into four subfamilies (type I to type IV) according to their phylogenetic relationships. Types I and II CHIs are bona fide catalysts having CHI enzymatic activity. Type I CHIs widely exist in vascular plants and are responsible for the production of plant flavonoids (27–29). Type II CHI proteins appear to be legume-specific and are involved in isoflavonoid production (27, 30). Type III CHIs are widely present in land plants and green algae, while type IV CHIs are restricted to land plants. Structural analysis showed that all CHIs share a similar backbone conformation (25, 30). However, type III and type IV CHIs do not possess bona fide CHI activity, which led to the renaming of both types of CHIs as CHI-like proteins (CHIL). Recently, type III CHI folds from Arabidopsis were shown to bind fatty acids in vitro, and play a role in fatty acid metabolism in planta (25). Type III CHIs are thus divided into three fatty-acid binding protein subfamilies (FAP1, FAP2, and FAP3). A loss-of-function mutation in type IV CHI from Japanese morning glory (Ipomoea nil) led to low amounts of anthocyanin, although the underlying mechanism remains unknown (31). Previous phylogeny and sequence analyses suggest that bona fide CHIs are diverged from type IV CHIs, which evolved from the common ancestor FAP3 (type III CHIs). However, the role of type IV CHIs in flavonoid biosynthesis during land plant evolution remains to be determined (25, 32, 33).

Here, we used DD104 yeast strain to characterize two hop CHIL genes (HICHIL1 and HICHIL2). We demonstrate that although lacking CHI activity, both CHILs play a critical role in demethylxanthohumol (DMX)/XN production. HICHIL2 enhanced the activities of CHS_H1 and PT1L through direct protein–protein interactions, suggesting that a functional metabolon composed of CHS,
CHIL2, and PT1L, directly efficient production of prenylchalcones in hop glandular trichomes. On the other hand, CHIL1 appears to bind and stabilize the ring-open configuration of chalcones during XN biosynthesis. Our study not only reveals the functions of non-catalytic CHIs in flavonoid biosynthesis and the evolutionary trajectory of non-catalytic CHIs in land plants, but also provides valuable insight into engineering heterologous systems to produce high-valued prenylchalcones at a large scale.

Results

Characterization of Two CHIL Genes from Hop Glandular Trichomes.

We have previously demonstrated that XN biosynthesis occurs predominantly in the glandular trichomes of female hop flowers (1, 21–23). Searching the hop trichome-specific EST library led us to find 260 ESTs representing CHI genes, which were among the most abundant unigenes in the library. Two ORFs of CHIL genes were isolated and designated as HICHIL1 and HICHIL2, which encode polypeptides of 214 and 209 amino acids, respectively. HICHIL1 shares 31% identity to FAP1 (At3g63170) from Arabidopsis thaliana (25) and 69% identity to a homologous protein from Cannabis sativa (GenBank accession no. JN679226), which is functionally uncharacterized to date (34). HICHIL2 shares 69.7% identity to AtCHIL (At5g05270), which functions with bona fide CHI (At3g55120) to promote flavonoid production in Arabidopsis (35). A maximum-likelihood phylogenetic tree of plant CHIs was constructed to analyze the evolutionary relationship among plant CHIs: CHIL1 belonged to the type III subfamily (FAP1 clade), whereas CHIL2 falls into the type IV subfamily (Fig. 2A). Sequence alignments showed that the conserved active residues in bona fide CHI sequences are missing in HICHIL1 and HICHIL2, suggesting both are noncatalytic CHIs (SI Appendix, Fig. S1). Biochemical assays, using NC and iso-liquiritigenin as substrates, at pH 6.4 and 7.5, supported this hypothesis: both CHIL1 and CHIL2 recombinant proteins did not exhibit CHI activity in vitro (SI Appendix, Fig. S2). This result is consistent with the fact that prenylchalcones, rather than prenylflavanones, are predominantly accumulated in hop trichomes (21, 22). Quantitative RT-PCR analysis showed that expression of both HICHIL1 and HICHIL2 was trichome-specific (Fig. 2B).

Subcellular localization experiments indicated that CHIL1 and CHIL2 were localized to the cytoplasm, where the CCL1, CHS_H1, and OMT1 enzymes of XN biosynthesis are also located (Fig. 2C). These results suggested that CHIL1 and CHIL2 are likely involved in XN biosynthesis.

CHIL1 and CHIL2 Exert Contrasting Effects on DMX Production. To explore the roles of HICHILs in DMX biosynthesis, we initially reconstructed the DMX pathway by coexpressing CCL1, CHS_H1, and PT1L (PT genes used in this study were Arabidopsis codon-optimized sequences) in the DD104 yeast strain (23). On feeding with 0.5 mM p-coumarate, the engineered strain harboring CCL1/CHS_H1/PT1L was able to produce DMX, whereas the strain harboring CCL1/CHS_H1/PT2 could not produce DMX (SI Appendix, Fig. S3). Moreover, addition of PT2 to the CCL1/CHS_H1/PT1L yeast strain did not further improve DMX production (SI Appendix, Fig. S3). These results indicated that H IPT1L alone is responsible for the prenylation step in the DMX pathway (SI Appendix, Fig. S3B). Because DMX is converted to 6PN (6-prenylnaringenin) and 8PN (8-prenyllarigenin) in this yeast system, we report total prenylated chalcone/flavanone (DMX, 6PN, and 8PN). When we further introduced CHIL2 in the DD104 yeast strain harboring CCL1/CHS_H1/PT1L, the CCL1/CHS_H1/PT1L/CHIL2 strain produced approximate 2.3-fold higher total DMX (4.59 ± 0.98 μmol/L/OD; n = 3) than CCL1/CHS_H1/PT1L (2.0 ± 0.31 μmol/L/OD; n = 3). Introduction of CHIL2 also increased naringenin (N/NC production by 1.3-fold (43.97 ± 5.8 μmol/L/OD vs. 34 ± 0.6 μmol/L/OD; n = 3) (Fig. 3). Unexpectedly, introduction of CHIL1 to the CCL1/CHS_H1/PT1L strain significantly reduced total DMX production by 30% (1.38 ± 0.32 μmol/L/OD; n = 3) (Fig. 3). We also checked the maximal production of N/NC and total DMX by adding p-coumarate to the culture (to final concentration 500 μM) every 24 h (29). The results showed that the DD104 yeast strain harboring CCL1/CHS_H1/PT1L/CHIL2 produced the N/NC and DMX at a relative flat rate (SI Appendix, Fig. S4 A–C). Additionally, most of the N/NC (72%) was secreted into the culture medium, whereas 90% of the DMX remained inside the yeast cells (SI Appendix, Fig. S4D).

Fig. 2. Characterization of HICHIL1 and HICHIL2. (A) Phylogenetic analysis of CHI/CHIL proteins from plants using the maximum-likelihood method. A total of 43 CHI/CHIL proteins were obtained from 13 species representative of plant evolutionary history. To simplify the classification of CHI/CHIL proteins, four clades (types I to IV) are shown here. Bootstrap values (based on 1,000 replicates) >70% are shown for corresponding nodes. The two hops CHILs are marked with red dots, and functionally identified CHILCHILs are marked with blue asterisks. Species abbreviations: At, Arabidopsis thaliana; Cs, Cannabis sativa; Gm, Glycine max; Hi, Humulus lupulus; In, Ipomoea nil; Lj, Lotus japonicus; Os, Oryza sativa; Ph, Petunia hybrida; Ps, Phascolarctos patens; Sm, Selaginella moellendorfii; Thy, Torenia hybrida; Vv, Vitis vinifera; Zm, Zea mays. Protein sequences used in this analysis are listed in Dataset S1. (B) Quantitative RT-PCR analysis of two CHIL genes in different tissues of hop plants. Transcript levels are expressed relative to GAPDH transcripts (n = 3). Cone bract, the glandular trichomes were removed; WAF, weeks after flowering. (C) Subcellular localization of XN-relevant enzymes in Arabidopsis leaf mesophyll protoplasts as revealed by laser confocal microscopy. Chloroplasts are revealed by red chlorophyll autofluorescence. (Scale bars, 5 μm.)
Type IV CHI-Folds Physically Interact with Plant CHSs to Enhance CHS Activity. The enhancing effect of CHIL2 on NC and DMX production leads to an assumption that CHIL2 might be physically interacting with other enzymes in the XN pathway. To test this hypothesis, we used the yeast two-hybrid (Y2H) technique to probe for interactions between CHIL2 and other enzymes in the XN pathway. The results showed that CHIL2 interact with CHS_H1, but not with other proteins in the XN pathway (Fig. 4A). We further confirmed the interaction between CHIL2 and CHS_H1 using luciferase complementation imaging (Fig. 4B) and coimmunoprecipitation (Co-IP) (Fig. 4C). As HIP1L is a membrane-bound protein, we used a split-ubiquitin MbY2H to test for possible interaction between CHIL2 and PT1L, and the results revealed that CHIL2 also physically interacted with PT1L protein (Fig. 4D). Furthermore, LUC activity detected in Nicotiana benthamiana leaves confirmed the interaction between PT1L and CHIL2 (Fig. 4E). We did not find any interaction between CHIL1 and CHIL2, PT1L, CCL1, CHS_H1, or OMT1 (Fig. 4A and D and SI Appendix, Fig. S5). We further evaluated the influence of CHIL2 on the enzymatic efficiency of CHS_H1 and PT1L through in vitro biochemical analysis. The combination of CHS_H1/CHIL2 had 1.5-fold higher NC production than CHS_H1 alone (SI Appendix, Fig. S6D). Microsome prepared from yeast harboring CHS_H1/PT1L/CHIL2 displayed 1.4-fold higher DMX production than from yeast harboring only CHS_H1/PT1L (SI Appendix, Fig. S6B). The conversion ratios of purified recombinant CHS_H1 (Vmax value) were increased by ~5.6-fold and 17.5-fold with CHIL2 for p-coumaroyl-CoA and malonyl-CoA, respectively, although the Km value also increased by fivefold (Table 1). PT1L/CHIL2 had both a higher conversion rate and a lower Km value than PT1L alone for both NC and DMAPP (Table 2).

Given that stress-protective flavonoids, type IV CHIs, and CHSs are widely distributed in land plants (25, 36, 37), we assume that type IV CHIs share the conserved function of increasing flavonoid production by binding and increasing the enzymatic efficiency of plant CHSs. To test this hypothesis, we probed the possible type IV CHI–CHS interactions in four plant species [Physcomitrella patens (Bryophytes), Selaginella moellendorffii (Lycophytes), Oryza sativa (monocots in Euphylllophytes), and Arabidopsis thaliana (dicots in Euphylllophytes)], which are located at the major nodes during plant evolution. The physical interactions were detected for all six tested type IV CHI–CHS pairs (there are two type IV CHIL genes in the P. patens and O. sativa genomes) (Fig. 4F). All tested type IV CHI proteins enhanced the CHS activity by 1.5- to 3-fold, as indicated by N/NC production in the engineered yeast system (Fig. 4G).

To test the function of CHIL2 in planta, we also generated transgenic Arabidopsis plants overexpressing CHIL2, PT1L, and CHIL2-PT1L in the n5-1 background [T7S (At3g55120), lacking a functional CHI protein; Ler ecotype], which accumulates approximately 60-fold more NC compared with Ler wild-type (SI Appendix, Fig. S7A). LC-MS analysis showed that the NC content was increased in CHIL2 overexpression lines by about 1.6-fold (SI Appendix, Fig. S7 B and C), indicating that CHIL2 increased CHS activity in planta. This result drove us to test whether HICHIL2 physically interacted with the CHS from A. thaliana (At5g13930) and other above-mentioned plant CHSs.

We found direct interactions between HICHIL2 and all tested CHS proteins from P. patens, S. moellendorffii, O. sativa, and A. thaliana (SI Appendix, Fig. S7D). However, we were unable to detect DMX or prenylated flavonoids in PT1L or CHIL2-PT1L overexpressing Arabidopsis lines, despite the detection of PT1L mRNA in these transgenic plants (SI Appendix, Fig. S8).

CHIL1 Binds to DMX and NC to Stabilize the Chalconoids’ Ring-Opened Structure. Because CHIL1 significantly reduced DMX production in engineered yeast cells (Fig. 3), although no physical interaction between CHIL1 and other XN-related proteins could be detected (SI Appendix, Fig. S5), it is possible that CHIL1 protein binds to DMX, similar to the way that AtFAPs bind with fatty acids in A. thaliana (25), and that the bound DMX cannot be released from this metabolite–protein complex by the extraction method used here. To test this hypothesis, we incubated purified His-tagged CHIL1 with the chemical extract (DMX/6PN/8PN) prepared from yeast harboring CCL1/CHS_H1/PT1L/CHIL2. Most of the DMX was eventually converted to 6PN and 8PN after incubation with His-tagged proteins for 8- to 4°C (see SI Appendix, Fig. S9 for the experimental workflow). However, CHIL1 protein enriched DMX levels by 14.2-fold [the ratio of (DMX)/(total DMX) in CHIL1-bound chemical mixture was 19.7%; n = 3, whereas the ratio of (DMX)/(total DMX) in solution was only 1.39%] (Fig. 5A). In contrast, no CHL2-associated DMX or 8PN were detected under the same incubation conditions, and the MagneHis Ni-Beads showed weak nonspecific binding activity to 6PN only (Fig. 5 A and B). Differential scanning fluorimetry assays (38) using purified HICHIL1 protein and standard chemicals further showed that CHIL1 protein binds DMX and NC with Kd (dissociation constant) values of 25.08 μM (n = 3) and 43.56 μM (n = 3), respectively (Fig. 5 C and D), whereas no such binding could be detected with N, 6PN, 8PN, or NC. We therefore conclude that the 6PN and 8PN detected in CHIL1-bound compounds are most likely converted from DMX during sample preparation given the known spontaneous isomerization of DMX in vitro. CHIL1 protein showed weaker binding activity to lauric acid (C12:0) and myristic acid (C14:0), than to longer-chain fatty acids (C16:0, C18:0, and C18:3) (SI Appendix, Fig. S10). It should be noted that both DMX (∆Tm max = 10.23 °C, n = 3) and NC (∆Tm max = 6.40 °C, n = 3) show a stronger stabilizing effect on CHIL1 protein than do lauric acid (∆Tm max = 5.0 °C, n = 2) or myristic acid (∆Tm max = 3.55 °C, n = 2). However, CHIL1 preferentially binds lauric acid (Kd = 11.81 μM, n = 2) and myristic acid (Kd = 3.45 μM, n = 2) compared with DMX (Kd = 25.08 μM,
Fig. 4. Direct interactions between type IV CHIs and CHS. (A) Protein–protein interactions between CHIL2 and CHS_H1, CHIL1, and CCL1 in a Y2H system. The selective medium [SD-Trp-Leu-His] containing 20 mM 3-amino-1,2,4-triazole (3AT) was used for selecting for the interacting proteins. The CHIL2 gene was inserted into pGBK7 vector (BD vector), and CHS_H1, CHIL1, and CCL1 were inserted into pGADT7 vector (AD vector). P.C., positive control; the pGBKT7-E5227 vector was used as BD vector, and pGADT7-T constructs used as AD vectors were provided by the manufacturer. (B) Direct interactions between HIL2 and CHS_H1 in N. benthamiana leaves. Luciferase image of N. benthamiana leaves coinfiltrated with the agrobacteria containing CHS_H1-nLuc and CHIL2-cLuc or CHIL2-nLuc/CHS_H1-cLuc combinations. (C) Reciprocal co-IP of MYC-tagged CHS_H1 and HA-tagged CHIL2 in yeast using HA- and Myc- antibodies. Total protein extracts were prepared from transgenic yeast strain harboring CHS_H1, CHIL2, and CCL1 constructs. (D) Type IV CHIL–CHS interactions in P. patens (there are two type IV CHIL genes in the P. patens genome). S. moellendorffii, O. sativa (there are two type IV CHIL genes in O. sativa genome), and A. thaliana. (E) Production of NNC by yeast strains harboring CHS alone or CHS–CHIL combination. Yeast strains harboring different gene combinations were grown in induction medium for 48 h before chemical extraction and analysis. Data are means ± SD for at least three independent clones (t test, **P < 0.01). N.C.1, negative control 1 (nLuc + cLuc); N.C.2, negative control 2 (Gene1-nLuc + cLuc); N.C.3, negative control 3 (nLuc + Gene2-cLuc); N.C.4, negative control 4 (Gene2-nLuc + cLuc); N.C.5, negative control 5 (nLuc + Gene1-cLuc).
generated V139F and A143F CHIL1 single mutants and V139F/A143F double mutant. Binding assays clearly showed that single CHIL1 mutant (CHIL1V139F and CHIL1A143F) had similar binding properties toward DMX (ΔTm\text{max} = 10.15 °C and 10.87 °C for CHIL1V139F and CHIL1A143F, respectively; n = 3) and NC (ΔTm\text{max} and Kd are 5.50 °C and 7.34 °C for CHIL1V139F and CHIL1A143F, respectively; n = 2), compared with those of wild-type CHIL1 (ΔTm\text{max} = 10.23 °C for DMX and 6.40 °C for NC; n = 3) (Fig. 6B). However, both DMX (ΔTm\text{max} = 5.26 °C, n = 3) and NC (ΔTm\text{max} = 4.70 °C, n = 2) show much weaker stabilizing effect on CHIL1V139F/A143F compared with CHIL1 (Fig. 6B), suggesting that V139 and A143 of CHIL1 have a joint effect on DMX/NC binding to CHIL1. It is noteworthy that CHIL1V139F shows higher affinity to DMX (Kd = 10.05 μM) and NC (Kd = 7.61 μM) than CHIL1 (Fig. 6B). Altogether, we tentatively designate these HICILL1 homologs as polyketide (a term covering flavonoid) binding proteins (BPB).

Discussion

It is well-known that flavonoids are ubiquitously present in land plants, where they may have played a key role in land colonization during plant evolution (39). CHS, the first enzyme of the flavonoid biosynthetic pathway, has been intensively studied at the biochemical and molecular levels during past three decades. Recently, a proteolytic regulator (At1g23390, encoding a Kelch domain-containing F-box protein) controlling CHS stability was functionally identified from A. thaliana (40). Here, we have identified another regulator of plant CHS, the type IV CHI-fold proteins, which physically interact with CHS to increase its activity, thereby enhancing flavonoid production. This characterization explains the previous observations that loss-of-function of type IV CHI from Japanese morning glory (I. nil) and Arabidopsis leads to decreased amounts of flavonoids (31, 35). Presumably through protein–protein interactions, the type IV CHI remodels the active-site cavity of CHS for synthesizing NC. The crystal structure of the HICILL2/HICHS_H1 will definitely elucidate the underlying molecular mechanism.

The CHS-enhancing property of the type IV CHI-fold proteins is conserved from mosses to flowering plants (Fig. 4). Although

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### Table 1. Kinetic parameters for CHS_H1 and CHS_H1/CHIL2 complex

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<th>Complex</th>
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<th>V_max (μmol/min/g)</th>
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<td>CHS_H1</td>
<td>p-Coumaroyl-CoA*</td>
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<td>Malonyl-CoA*</td>
<td>10.06 ± 2.99</td>
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<td>CHS_H1/CHIL2</td>
<td>p-Coumaroyl-CoA*</td>
<td>26.57 ± 3.78</td>
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<td>Malonyl-CoA*</td>
<td>59.74 ± 8.49</td>
<td>4.2 ± 0.3</td>
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Two micrograms of purified recombinant CHS_H1 were used in each CHS_H1 assay; and 2 μg of purified recombinant CHIL2 (CHS_H1:CHIL2 = 1:1) were used in each CHS_H1/CHIL2 assay.

* A fixed concentration of 150 μM malonyl-CoA was used as substrate.

† The data are presented as means ± SD (n = 3).

‡ A fixed concentration of 40 μM p-Coumaroyl-CoA was used as substrate.

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### Table 2. Kinetic parameters for PT1L and PT1L/CHIL2 complex

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<td>PT1L</td>
<td>NC*</td>
<td>5.74 ± 0.68</td>
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<td>DMAPP\†</td>
<td>75.12 ± 15.7</td>
<td>0.13 ± 0.02</td>
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<tr>
<td>PT1L/CHIL2</td>
<td>NC*</td>
<td>5.01 ± 0.43</td>
<td>0.12 ± 0.01</td>
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<td></td>
<td>DMAPP\†</td>
<td>62.78 ± 6.76</td>
<td>0.23 ± 0.01</td>
<td>0.0037</td>
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Total membrane-bound proteins (10 μg in each assay) were prepared from the yeast harboring PT1L or PT1L/CHIL2.

* A fixed concentration of 50 μM DMAPP was used as substrate.

† The data are presented as means ± SD (n = 3).

‡ A fixed concentration of 200 μM NC was used as substrate.
Identification of DMX binding of CHIL1. (A) Analysis of ligands associated with purified His-tagged HICHIL1 protein (linked to MagneHis Ni-Particles) separated and detected by LC-QQQ-MS/MS (MRM condition for DMX/6PN/8PN: 341.0 → 165.0). MagneHis Ni-Beads alone were used as negative controls in these assays. All samples, including the chemical extract only, were incubated at 4 °C for 8 h before LC-MS analysis (for details, see SI Appendix, Fig. S9). (B) Relative quantification of DMX in protein-bound and supernatant fractions of different CHIL-small-molecule interaction assays. N.D., not determined due to low content of DMX in the samples (t test, **P < 0.01). (C) Representative dose–response curves for HICHIL1 generated using thermal shift response to increasing DMX concentration. \( \Delta T_{m_{\text{max}}} \) and \( K_D \) values were calculated as 10.23 °C and 25.08 μM, respectively (n = 3). (D) Representative dose–response curve for HICHIL1 generated using thermal shift response to increasing NC concentration. \( \Delta T_{m_{\text{max}}} \) and \( K_D \) values were calculated as 6.40 °C and 43.56 μM, respectively (n = 3). (E) Homology model of HICHIL1 (marine) with docked DMX ligand (yellow sticks) overlaid with AtFAP1 (PDB ID code 4DOO; magenta) bound to lauric acid (green sticks). The homology model was built using AtFAP1 as a template. (F) Zoomed-in view of HICHIL1 with docked DMX, showing relevant protein side chains as gray sticks. Selective hydrogen atoms are shown in purple, H-bonding interactions as dotted black lines, and potential \( \pi \)-stacking or cation–\( \pi \) stabilization interactions as solid black arrows. Val139 and Ala143 are highlighted in red due to their important role in DMX binding.

Fig. 5. Identification of DMX binding of CHIL1. (A) Analysis of ligands associated with purified His-tagged HICHIL1 protein (linked to MagneHis Ni-Particles) separated and detected by LC-QQQ-MS/MS (MRM condition for DMX/6PN/8PN: 341.0 → 165.0). MagneHis Ni-Beads alone were used as negative controls in these assays. All samples, including the chemical extract only, were incubated at 4 °C for 8 h before LC-MS analysis (for details, see SI Appendix, Fig. S9). (B) Relative quantification of DMX in protein-bound and supernatant fractions of different CHIL-small-molecule interaction assays. N.D., not determined due to low content of DMX in the samples (t test, **P < 0.01). (C) Representative dose–response curves for HICHIL1 generated using thermal shift response to increasing DMX concentration. \( \Delta T_{m_{\text{max}}} \) and \( K_D \) values were calculated as 10.23 °C and 25.08 μM, respectively (n = 3). (D) Representative dose–response curve for HICHIL1 generated using thermal shift response to increasing NC concentration. \( \Delta T_{m_{\text{max}}} \) and \( K_D \) values were calculated as 6.40 °C and 43.56 μM, respectively (n = 3). (E) Homology model of HICHIL1 (marine) with docked DMX ligand (yellow sticks) overlaid with AtFAP1 (PDB ID code 4DOO; magenta) bound to lauric acid (green sticks). The homology model was built using AtFAP1 as a template. (F) Zoomed-in view of HICHIL1 with docked DMX, showing relevant protein side chains as gray sticks. Selective hydrogen atoms are shown in purple, H-bonding interactions as dotted black lines, and potential \( \pi \)-stacking or cation–\( \pi \) stabilization interactions as solid black arrows. Val139 and Ala143 are highlighted in red due to their important role in DMX binding.

phylogenetic analysis suggested that the type IV CHI-fold proteins were probably the ancestor of plant functional CHIs, the function of type IV CHI-fold proteins remained unknown (25, 32, 33). Based on our data, we propose that binding with CHS is a conserved function for all plant CHI-fold proteins, type I and type II CHI-fold proteins inherited this trait from type IV CHI-fold proteins besides the gained CHI activity during enzyme evolution. The physical interaction between bona fide CHI and CHS proteins may bind with enzymes downstream of CHS in a species-dependent manner; this needs to be experimentally validated.

Meanwhile, CHIL1 binds and stabilizes the ring-open conformation of NC and DMX, the intermediates of XN biosynthesis. The DMX-binding ability of CHIL1 and the DMX metabolism presented here are consistent with the chemical phenomenon observed in hop glandular trichomes: high accumulation of DMX and XN, with almost no N/NC being detected
In conclusion, we have functionally identified two CHIL homologs, HlCHIL1 (69% identity) and CsaCHIL, which play a significant role in polyketide biosynthesis to result in high production of polyketides in a specific plant cell/tissue/organ.

Fig. 6. Sequence analysis of HICHIL1 homologs and chemical binding identification of CHIL1 mutants. (A) Thirty-three HICHIL1-close proteins (>50% identity) were added for phylogenetic analysis. The GenBank number for each HICHIL1-close protein is provided in parenthesis, next to the Latin name of the plant species. Bootstrap values (based on 1,000 replicates) >70% are shown for corresponding nodes. HICHIL1 and CsCHIL are marked in red dots. Twenty residues are added for the phylogenetic analysis. The GenBank number for each HICHIL1-close protein is provided in parenthesis, next to the Latin name of the plant species. Bootstrap values (based on 1,000 replicates) >70% are shown for corresponding nodes. HICHIL1 and CsCHIL are marked in red dots. Twenty residues are added for the phylogenetic analysis. (B) Representative dose–response curve for CHIL1, CHIL1V139F, CHIL1V143F, and CHIL1V139F/A143F generated using thermal shift response to increasing DMX or NC concentration.

(21, 22). The structural information and sequence analysis has led to the discovery of a protein clade (PBP) with unprecedented functions: HICHIL1 homologs probably bind with the products of other type III polyketide synthases, at least those that contain an aromatic ring derived from the condensation of three molecules of malonyl CoA (Fig. 5). CsCHIL (from C. sativa, Cannabaceae) is the closest homolog of HICHIL1 (69% identity), and is specifically expressed in hemp glandular trichomes (34). Two polyketides, Δ2-tetrahydrocannabinolic acid (THCA) and cannabidiolic acid (CBDA), are synthesized and stored at high levels in hemp glandular trichomes. THCA and CBDA, like DMX, are unstable and will be nonenzymatically converted to the decarboxylated forms, Δ2-tetrahydrocannabinol and cannabidiol, respectively. We therefore hypothesize that CsCHIL probably binds with THCA and/or CBDA to stabilize these compounds in hemp glandular trichomes. Furthermore, the endogenous content of free polyketides will be accordingly decreased due to increased protein-bound forms, which can alleviate the feedback inhibition of polyketide biosynthesis.
under flask-shake conditions, which will be a starting point for producing these valuable pterylchalcones at large scale using microbial cell factories.

Materials and Methods

Plant Materials, RNA Analysis, and Chemicals. The growth of H. lupulus cv. Nugget, EST sequence analysis, RNA isolation, and cDNA preparation from hop tissues were performed as described previously (1). A. thaliana (Col-0 ecotype) and N. benthamiana plants for transient transformation were maintained in a greenhouse under 16-h light/8-h dark, 22 °C conditions. All available commercial chemicals used in this study were purchased from Sigma-Aldrich, except for NC, which was purchased from ChromaDex. PIVP (phosphinoisovaleryl-proteins), or 150 μmol/mL-CoA was used in the standard PT reactions (200 μL) containing 10-μg membrane proteins and DMAPT at a series of concentrations. The enzymatic products were extracted and quantified as previously described (23). The apparent Km value was calculated using Hanes plots (Hyper32, v1.0.0).

ChI assays were carried out as described previously (27, 47), using NC and isoleucine/arginine as substrates, at pH 6.4 and 7.5. Two microliters of each enzymatic reaction, after filtered through a 0.22-μm syringe filter, was loaded on a 1290 Infinity LC pump coupled to a 6495 triple quadrupole mass spectrometer equipped with a dual electrospray ion source operated in positive mode (LC-QQQ-MSMS; Agilent). Chromatographic separation was performed on a ZORBAX Extend C18 column (50 mm × 2.1 mm id, 1.8 μm; Agilent). Gradient condition of the mobile phase (solution A is water and solution B is methanol; flow rate is 0.35 mL/min) was set as follows: 0–6 min, a linear gradient of from 40% of B to 60% of B; 6.0–6.5 min, a linear gradient of from 60% of B to 98% of B; 6.5–7.5 min, 98% of B; then the system was equilibrated using the initial condition (40% of B) for 5 min before the next sample injection.

Yeast Two-Hybrid Assays. Yeast two-hybrid assays were based on the Matchmaker GAL4 two-hybrid system (Clontech). CHIL2 was inserted into pGBK7 vector, and CCL1, CHS_H1, CHIL1 were inserted into pGADT7 vector. Constructs were cotransformed into the yeast strain Y2H Gold using a PCR-mediated method (22). The transgene was confirmed by growth on SD-Leu-Trpplates. To assess protein interactions, the transformed yeast was suspended in liquid SD-Leu-Trp medium and cultured to OD600 = 1.0. Ten microliters of suspended yeast was spread on SD-/His-/Leu-Trp medium with 20 mL 3-AT. Interactions were observed after 3 d of incubation at 30 °C. The split-ubiquitin Matl/YTH assays were performed with a DUAL membrane kit following the manufacturer’s instructions (Dualsystems Biotech). PTIL was cloned into the bait vector pTM4, and CHIL1, CHIL2, CHS_H1, and CCL1 were cloned into the pDlex-Nx vector. Constructs were cotransformed into the yeast strain DSY-1. The experiments were repeated for at least three times. The primers used in this study are listed in Table S1, Table S2.

Luciferase Complementation Imaging Assay. CHIL genes (AtCHIL, OsCHIL1, OsCHIL2, SmCHIL1, PpCHIL1, and PpCHIL2) were fused to the N- or C-terminal part of LUC to generate the corresponding nLUC and cLUC constructs. Agrobacterium tumefaciens GV3101 containing the corresponding nLUC and cLUC fusion constructs were simply mixed with P19, then infiltrated into leaves of N. benthamiana for transient expression. LUC activity was detected after 48–72 h. The luciferase complementation imaging assays were performed as previously described (48), using three biological replicates, by infiltration of leaves on separate plants. Primers used for the vector construction are shown in Table S1, Table S2.

Co-IP Assays. N-terminal HA-tagged CHIL2 and Myc-tagged CHS_H1 were cloned into the pEYF4 vector and pESC-His vectors respectively. HA-pESC-TRp and Myc-pESC-His plasmids which expressed HA/myc tag alone were constructed as negative controls using a PCR-mediated method (for primer information, see Table S2). The transformed yeast cells (DSY-1 strain) were harvested after galactose induction until the OD600 value reached 1.0, and disrupted with glass beads with the Mini-Bead-Beater (Biospec Products) in an extraction buffer (2 mL/g cells) containing 50 mL Tris-HCl (pH 7.5), 1 mL EDTA, 1% Triton X-100, 1 mg phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 1 μg/mL pepstatin. Glacial beads and cell debris were removed by centrifugation at 12,000 × g for 10 min. The supernatant was incubated with the first antibody (anti-Myc) at 4 °C overnight with shaking, followed by incubation with Protein G Agarose beads for an additional 2 h. The beads were washed twice with extraction buffer supplemented with 300 mL NaCl and then the proteins were released by incubation for 10 min in SDS sample buffer at 98 °C and analyzed with immunoblotting using an anti-HA antibody.

Chemical Measurements in the Transgenic Arabidopsis Lines. Empty pGreen binary vector (control) (49), CHIL2, PTIL, and CHIL2-PTIL were introduced into rts-1 mutant Arabidopsis using the floral-dip method (50). Two-week-old seedlings, grown on 1/2 MS plates, were ground into a fine powder in liquid nitrogen, of which 200-μg fresh weight equivalents was extracted with 1.6 mL 50% ethyl acetate. After centrifugation for 10 min, the supernatant was evaporated to dryness and the residue dissolved in methanol and filtered through a 0.22-μm syringe filter. NINC measurements were conducted using above-mentioned LC-QQQ-MS/MS program.
Protein–Metabolite Interaction Assay. CHIL genes were subcloned into the pEasy-Blunt E2 Expression vector (Transgen). All constructs were transformed into Escherichia coli BL21 (DE3) cells for prokaryotic expression, and the resulting His-tagged fusion proteins were purified using Ni-NTA affinity chromatography. Quantification and evaluation of the relative purity of the recombinant proteins was performed using SDS/PAGE with BSA as a standard. The in vitro reaction buffer contained 50 mM Tris-HCl, pH 7.5, 20% methanol, 8 μL yeast extract (final concentration around 200 μM NNC, 5 μM DMX), and 30 μg purified protein in a final volume of 500 μL. After incubation at 37°C for 8 h, the protein in the buffer was extracted using Magneti His Protein Purification System (Promega). The compounds were extracted from the supernatant using ethyl acetate, while the Magneti His Beads were washed twice with 50 mM Tris-HCl (pH 7.5), and the bound chemicals were eluted with ethyl acetate. The chemicals obtained from the supernatant and Ni-Beads were analyzed by LC-QQQ-MS/MS, as described above.

Differential Scanning Fluorimetry Assays. The parameters (Kf and ΔTm) of CHIL1 binding with DMX or NC were measured using a StepOne real-time PCR system (Applied Biosystems) as previously described (25, 38). The reaction assays contained 5.0 μL of Protein Thermal Shift Buffer, 2.5 μL of 8× Diluted Protein Thermal Shift Dye, 5 μM purified HICHIL protein, in a final volume of 20 μL. The chemicals used in these assays were serially diluted from 250 μM to 0.12 μM (total 12 points), and Kf and ΔTm were calculated using GraphPad Prism 5 from at least three biological replicates.

Homology Modeling and Docking Simulations. All structure modeling was performed using the Maestro software package from Schrödinger LLC. The homology model was generated using Prime with AutoFAP1 (PDB ID code 4DOO) as the template model. Hydrogen atoms were added to the model and minimized before docking simulations. Potential binding pockets were identified with SiteMap and the DMX ligand was prepared using LigPrep. Individual DMX docking poses were obtained and scored using Glide, then assessed for selection of the best fit. PyMOL was used for the generation of the final model images.

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